Boston University
Graduate School

Dissertation

The Use of Adjuvants in the Production of Rh Antisera in Animals

by

Harriet Sommer Shatin

(B.A., Brooklyn College, 1939; M.A., University of Pennsylvania, 1940)

Submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
1949

Approved
by

First Reader David L. Belding
Professor of Bacteriology and Experimental Pathology

Second Reader William C. Boyd
Associate Professor of Biochemistry

TOPICAL OUTLINE

Page

INTRODUCTION

The Problem

1. Need for Rh-Hr antisera-use in the diagnosis of Rh types of individuals ---------- 1

   a. Transfusions ---------- 2

      (1) Immunization

      (2) Reaction after previous transfusions

   b. Pregnancies ---------- 3

   c. Typing of infants ---------- 3,4

   d. Medico-legal problems ---------- 4

   e. Genetical studies of familial groups --------- 4

   f. Anthropology ---------- 4

2. History

   a. Discovery of the Rh antigens ---------- 4,5,6

   b. Lines of investigation

      (1) Nomenclature ---------- 6,7

      (2) Rh-Hr antigens ---------- 6-20

         (a) Inheritance of the Rh-Hr antigens --- 7

            (1) Wiener's theory of multiple allelomorphs ---------- 7,8

            (2) Fisher's theory of three closely linked pairs of genes ---------- 8

         (b) Kinds

            (1) Rhesus antigens ---------- 9,10

Page

(2) Human antigens ---------- 10-15

(a) D (d) C (g) c

(b) D^u (e) C^w (h) c^v

(c) d (f) C^u (i) E

(j) e

(c) Location of Rh-Hr antigens ---------- 16-18

(d) Chemical studies on the Rh-Hr antigens -- 18-20

(3) Rh antisera ---------- 20-

(a) Sources

(1) Human ---------- 20-27

(a) Conditions producing Rh antibodies ---------- 20-24

1. Pregnancy

2. Incompatible transfusion

3. Hyperimmunization

4. Voluntary active immunization

5. Influence of heterospecific immunization

(b) Transmission of fetal cells into the mother ---------- 24

1. Wiener's theory

2. Levine's theory

(c) Genetic predisposition toward antibody production --------- 25

1. Haldane's gene-permeability of placenta

2. Levine's theory - general genetic expression

Page

3. Wiener's "K" gene

(d) Preparation of Rh antisera --- 25-26

1. Sources of potent sera

2. Specifications

3. Elimination of normal iso-agglutinins

4. Concentration of anti-bodies

(e) Advantages and disadvantages of human Rh antiserum ------- 26,27

(2) Animal

(a) Rhesus erythrocytes as antigen 28-35

1. Landsteiner and Wiener - rabbits and guinea pigs -- 28,29

2. Davidson and Toharsky - guinea pigs and rabbits -- 29,30

3. Fisk and Foord - guinea pigs ---------- 30-32

4. Gallagher and Jones - guinea pigs and rabbits -- 32,33

5. McIvor and Lucia - guinea pigs ---------- 33

6. Ecker, MacFarlane and Laipply - guinea pigs ---- 33,34

7. Homburger - guinea pigs and rabbits ---------- 34

8. Scherer - rabbits -------- 34,35

(b) Human erythrocytes as antigen - 35-37

1. Levine and Stetson - rabbits ---------- 35

2. Levine and Polayes - guinea pigs and rabbits --------- 35

Page

3. Gallagher and Jones - guinea pigs ---------- 36

4. Carter - guinea pigs ---- 36,37

(c) Human stroma as antigen ----- 37

Gallagher and Pillischer - guinea pigs

(d) Extracts from human erythrocytes as antigen ---------- 37,38

Carter - guinea pigs and rabbits

(e) Advantages and disadvantages of animal antisera ---------- 38

(b) Types of Rh antibodies ---------- 38-40

(1) Agglutinins ---------- 39

(2) Blocking antibodies ---------- 39,40

(3) "Cryptagglutinoids" or "developing" antibodies ---------- 40

(c) Technical Methods ---------- 40

(1) Agglutinins ---------- 40,41

(a) Landsteiner and Wiener

(b) Modifications - Wiener

(2) Blocking antibodies ---------- 41

(a) Inhibition of indirect blocking test

1. Wiener ---------- 41

2. Race ---------- 41,42

3. Diamond and Abelson ------ 42,43

(3) Slide tests

(a) Diamond and Abelson's -------- 43,44

Page

(b) Simmons' ---------- 44

(4) Use of various colloidal media instead of saline

(a) Adult serum or plasma ------- 44-46

(b) Serum of premature and newborn infants ---------- 46

1. Lubinski ---------- 46

2. Levine ---------- 46

3. Gurevitch, Polishuk and Hermoni ---------- 46,47

4. Witebsky, Rubin and Blum - 47-50

(c) Albumin - Diamond and Denton - 50

(d) Cerebrospinal fluid - Jacobowicz and Bryce ---------- 51

(e) Gelatin - Diamond and Denton -- 52,53

(f) Glue - Wheeler and Scholl -----

(5) Coombs test - "developing" test ---- 53-55

(a) Direct test ---------- 53

(b) Indirect test ---------- 54

1. Method of testing

2. Preparation of rabbit anti-human-globulin serum

3. Fractions of human serum as antigens

4. Information as to the nature of "incomplete" antibodies

a. Serological basis

b. Chemical basis

Page

5. Effect of rabbit anti-globulin sera to species other than man

(6) Elution of antibody from sensitized cells ---------- 57

(a) Agglutinins ---------- 57,58

1. Haberman and Hill

2. Carter and Loughrey

3. Wheeler and Scholl

(b) Blocking antibodies - Wheeler and Scholl ---------- 58

(7) Capillary tube methods ---------- 58,59

(a) Chown ---------- 58

(b) Berlin ---------- 58

(c) Krieger and Bryce ---------- 58

(8) Hemolytic activity of agglutinins and blocking antibodies *in vitro* -- 59

(d) Nature of Rh antibodies---------- 59-67

(1) Chemical nature ---------- 59-61

(a) Chemical studies ---------- 59,60

(b) Serological studies ---------- 60

(c) Electrophoretic studies ------ 61

(d) Electric charges -- ---------- 61

(e) Molecular weight ---------- 61

(f) Ultracentrifugal studies ----- 61

(2) Physical characteristics ---------- 62,63

(a) Effect of heat ---------- 62

Page

(b) Effect of pressure ------- 62

(c) Effect of photo-oxidation 62,63

(d) Effect of merthiolate ---- 63

(e) Effect of saline --------- 63

(f) Effect of various colloidal media ---------- 63

(3) Immunological characteristics - 63,64

(a) Species in which found --- 63

(b) Time of appearance ------- 64

(c) Persistence of antibodies 64,65

(4) Serological characteristics --- 64,65

(a) Rapidity of reaction with red cells ---------- 64,65

(b) Reversibility of attachment to red cells ---------- 65

(c) Strength of clumping ----- 65

(d) Specificity of clumps ---- 65

(e) Reaction with cells in the presence of complement --- 65

(5) Clinical characteristics ------ 66

(a) Permeability of placenta -- 66

(b) Importance in the production of erythroblastosis fetalis 66

(6) Evidence for a third order antibody ---------- 66,67

(e) Concentration of antibodies --------- 68

(f) Specificity of Rh antibodies -------- 68,69

Page

PRESENT INVESTIGATION

1. Rationale ---------- 70-72

a. Present status of the Rh antisera - summary -------- 70

b. Use of adjuvants in immunization procedures -------- 70,71

(1) Purposes ---------- 70,71

(2) Examples ---------- 71

(3) Application of the adjuvant method of immunization to the present problem ---------- 71,72

2. Experimental

Experiment I Macacus rhesus erythrocytes as the antigen 72-74

Experiment II Human Rh positive erythrocytes as the antigen ---------- 74-79

(1) Selection of animals ---------- 74

(2) Preparation of antigens ---------- 74,75

(3) Preparation of emulsions ---------- 75

(4) Injection schedule ---------- 75

(5) Bleeding schedule ---------- 75,76

(6) Testing of sera ---------- 76-79

(a) For agglutinins ---------- 76,77

(b) For blocking antibodies ---------- 77,78

(c) For specific antibodies ---------- 78,79

(7) Criteria for adequate and weak antibodies ----- 79

3. Results

Experiment I ---------- 79

Experiment II ---------- 80-82

(1) Agglutinins ---------- 80-82

Page

(2) Blocking antibodies ---------- 82

(3) Coombs antibodies ---------- 82

DISCUSSION 83-87

SUMMARY 88-89

LIST OF TABLES

TABLE I - Rh-Hr Antigens and Antibodies

TABLE II - The 36 Possible Rh Genotypes, Showing the Reaction of Each with Each of the Six Antisera

TABLE III - Experiment I Material Injected per Animal

TABLE IV - Experiment II Groups of Guinea pigs Used

TABLE V - Experiment II Material Injected per Animal

TABLE VI - Experiment II Distribution of Adequate and Suggestive Rh Antisera

TABLE VII - Experiment II Summary of Strength of Rh Agglutinin Reactions of Guinea Pigs' Antisera

INTRODUCTION

The Problem

Since the discovery of the Rh antigens 9 years ago,[77] and subsequent discovery of the Hr antigens,[85] evidence indicating the importance of these antigens has been accumulating rapidly. Rh-Hr typing is important in clinical medicine, in medico-legal situations, in the genetical study of familial groups, and in anthropology.

The need for supplies of Rh antisera has taken its place next to that for anti-A and anti-B blood grouping sera. The latter problem has been pretty well solved by the use of serum from human volunteers of groups A and B whose anti-B and anti-A agglutinins have been stimulated to increased strength and avidity by the injection of A and B group substances.[160] A number of workers have attempted to supply the needed anti-Rh sera by centralized collection and distribution of potent sera from Rh-sensitized women, particularly mothers of erythroblastotic infants. Nevertheless, the supply, particularly that of the subtypes, is far from adequate, is sometimes variable, and the sera are expensive. It is obvious that methods of producing a constant source of Rh antisera in quantity would be of great importance. The present investigation summarizes the results of an intensive study of possible methods of producing

such sera in animals.

Clinically, the importance of the Rh-Hr antigens lies in their antigenicity for humans. The production of the individual antibodies depends on two factors: the potency of the antigen, and the reactivity of the individual to the antigen. It is customary to define an Rh negative person as one in whom the Rh_o(D) antigen is missing.[145] The 3 clinical situations where it is necessary to know the Rh type arise in transfusions, pregnancy, and in infants with evidence of erythroblastosis.

Rh negative males or females may be immunized by primary transfusions[4,11,29,30,31,39,63,84,102,132] so that any further injections of Rh positive blood would cause a transfusions reaction. Although more than 1 transfusion of Rh positive blood is usually needed to initiate antibody formation, there is wide individual variation. Sensitization may be produced by only 1 transfusion.[4] Transfusion reactions have also been produced in response to the other Rh and Hr antigens.[31,126,129] In these cases it is advisable to subtype the cells of the recipient, and those of the donor to pick out compatible blood for subsequent transfusions. Also, Rh negative males and females may have been transfused with Rh positive blood in the past, in which case the use of an

Rh positive blood may cause an immediate transfusion reaction.[85]

In pregnancies sensitization of females to the Rh antigens by transfusions may cause hemolytic disease of the first born.[91,94] Erythroblastosis fetalis is most frequently caused by Rh incompatibility between mother and infant. The role of the Rh factor is highly significant. Only 15 per cent of the women in the general population are Rh negative, whereas among mothers of erythroblastotic infants, 90 per cent are Rh negative.[83,89] Wallerstein[135] stated that D is responsible for 92 per cent of the cases. The rest result from iso-immunization against the A and B group substances,[108] Hr factors,[92] or Rh subgroups. In most instances the first infant is not affected. However, if the mother has been transfused previously with incompatible blood, she may never be able to produce a normal infant. The prognosis for future pregnancies of women with erythroblastotic infants may be made by determining the Rh type of the father.[31] If he is heterozygous for the antigen against which she has produced antibodies, there is a 50-50 chance that a subsequent pregnancy will be normal, but if he is homozygous there is little chance.

If an infant shows evidence of erythroblastosis, anemia,

or jaundice, it is necessary to type the infant's blood prior to transfusion. Generally Rh negative blood is preferred, but it is also necessary to type the infant's blood in case the sensitization is due to one of the Rh subtypes or the Hr antigens.

In addition to its clinical importance, Rh typing is useful in medico-legal problems[147,150] and in familial[100,113,115,128,141,144] and anthropological[10] studies.

History

In 1923 Ottenberg[107] and in 1938 Darrow[27] suggested that the clinical condition of erythroblastosis fetalis was caused by an antigen-antibody reaction in the bloodstream of the fetus. Landsteiner, Levine and Janes[76] in 1928, and Neter[106] in 1936 described the appearance of intragroup atypical agglutinins several weeks after transfusions. In 1939 Levine and Stetson[81] reported the occurrence of an isoagglutinin in a female who had given birth to a macerated stillborn fetus, and had experienced a transfusion reaction when transfused for the first time with blood from her husband of the same group. Their explanation of the patient's reaction to her first transfusion was that the blood and/or tissues of the fetus contained antigens inherited from the father that were not present in the mother, and that isoimmunization resulted

from transplacental transfer of fetal red cells and/or tissue cells into the maternal circulation.

In 1940 three papers appeared which pointed to the discovery of a new antigen present in human erythrocytes. Landsteiner and Weiner[77] reported the finding of an agglutinin in the serum of rabbits that had been immunized with cells from rhesus monkeys. After they had removed agglutinins reacting nonspecifically with human cells and those reacting with the agglutinogen M, an agglutinin remained which reacted independently of A and B, M and N, and P, with 39 of 45 human bloods. Wiener and Peters[138] observed 3 patients in whom transfusions with blood of the homologous group resulted in hemolytic reactions. In 2 of the patients the agglutinins were found to be identical with each other, and to correspond with Landsteiner and Wiener's anti-rhesus agglutinin. Eighty-five per cent of the white population was found to be Rh positive. In reviewing intra-group transfusion reactions, Wiener and Peters noted that every recorded instance of reaction to the first transfusion occurred with intra- or postpartum patients. Their observation supported Levine and Stetson's hypothesis that iso-immunization is brought about during pregnancy. Finally, Levine and Katzin reported 7 transfusion cases in which both wife and husband were of the same blood group, but where atypical maternal antibodies acted

on the cells of the husband. No history of previous blood transfusion was found in any of the cases. The agglutinins of 1 serum were found to correspond to the Rh agglutinins of Landsteiner and Wiener's rabbit antiserum. Additional evidence for the relationship of miscarriages, stillbirths, and erythroblastosis fetalis to iso-immunization during pregnancy was presented by Levine, Katzin and Burnham[82] in 1940, and by Levine and Polayes[87] in 1941.

Thus, the demonstration of the new antigen, Rh, was the result of the linking of 3 lines of investigations. The first was the study of intra-group transfusion reactions in individuals who had received multiple transfusions. The second was the investigation of intra-group transfusion reactions in females not previously transfused, and associated with disease of the fetus. The third was the production of an agglutinin in animals immunized with the erythrocytes of the rhesus monkey. Agglutinins produced under all 3 conditions were found to be identical.

Rh-Hr Antigens

Nomenclature.- Before entering into a discussion of the Rh-Hr antigens and their antibodies, it is necessary to identify the Rh-Hr antigens. In discussing the genes, antigens, and antibodies, two systems of nomenclature are in use. One

is the Weiner system[154] using Rh and Hr designations. The other is the Race-Fisher system[114,118,119] based on the letters C, D, and E. Castle, Wintrobe and Snyder[18] recommended that Weiner's terminology be used followed by the Race-Fisher terms in parentheses. Since this is very cumbersome the terminology which most clearly depicts the situation will be used in the present paper. As indicated in Table I there are 3 Rh antigens and 3 Hr antigens, with their corresponding antibodies. This simplification does not take into account the variants of the antigens, which are discussed below.

Inheritance.- Familial studies have disclosed that the presence of the Rh-Hr antigens in the red cells is genetically determined. No antigen is present in the blood of a child that is not present in the blood of its parents. As with the MN antigens in man, genes determining presence of the Rh and Hr antigens are of equal dominance. In other words, if an individual is heterozygous for C, his red cells contain both C and c antigens. These cells will react with both anti-C and anti-c sera. The presence of C will not mask that of c, nor will the reverse situation occur. The same holds true for the other two pairs of antigens, D-d and E-e.

There are two main theories concerning the inheritance of the Rh-Hr antigens. Wiener's theory[146] of multiple allelo-

TABLE I

Rh-Hr Antigens and Antibodies

	Antigen		Antibody		
Rh + antigens and their antibodies	Wiener	Race-Fisher	Wiener	Race-Fisher present	Race-Fisher orig.
	rh'	C	anti-rh'	anti-C	⌐
	Rh^{o}	D	anti-Rh^{o}	anti-D	△
	rh"	E	anti-rh"	anti-e	⊢
Rh negative antigens and their antibodies	Hr'	c	anti-Hr'	anti-c	⌒
	Hr^{o}	d	anti-Hr^{o}	anti-d	[illegible]
	Hr"	e	anti-Hr"	anti-e	[illegible]

morphs postulates 8 allelomorphic genes, each of which determines 1 of the 8 possible combinations of Rh antigens that can be present in human red cells. Since one gene is inherited from each parent, 2 of these 8 genes are present in an individual. The possible allelomorphs are: r,r',r", R^o,R^1,R^2,r^y,R^z. Fisher's theory[117,119] is that the Rh type of an individual depends on a series of 3 closely linked pairs of genes, C-c, D-d, and E-e. Each gene determines the inheritance of 1 antigen. Since 1 gene of each of the 3 pairs is transmitted from each parent, an individual may be homo- or heterozygous for each pair of antigens. That is, he may inherit CC, Cc, or cc, DD, Dd, or dd, and EE, Ee, or ee. The genotypes, phenotypes, and their serological reactions are given in Table II.

The substantiation of one theory or the other hinges upon the question of whether independent segregation of the 3 genes occurs. If independent segregation of the 3 genes were demonstrated, then Fisher's theory would be substantiated. If, however, this were shown not to occur, then Wiener's theory would not be contradicted. For example, if CDe x cde parents could produce a cDe offspring, this would lend credence to Fisher's theory. However, if the 3 antigens in the parent were always found to be inherited as a unit in the offspring, Wiener's theory would remain uncontested. To date,

TABLE II

The 36 Possible Rh Genotypes, Showing the Reaction of Each With Each of the Six Antisera

Genotype	Reaction with serum					
	anti-C	anti-D	anti-E	anti-c	anti-d	anti-e
CDE/CDE	+	+	+	—	—	—
CDE/CDe	+	+	+	—	—	+
CDE/Cde	+	+	+	—	+	+
CDE/CdE	+	+	+	—	+	—
CDE/cDE	+	+	+	+	—	—
CDE/cdE	+	+	+	+	+	—
CDE/cDe	+	+	+	+	—	+
CDE/cde	+	+	+	+	+	+
CDe/CDe	+	+	—	—	—	+
CDe/Cde	+	+	—	—	+	+
CDe/CdE	+	+	+	—	+	+
CDe/cDE	+	+	+	+	—	+
CDe/cdE	+	+	+	+	+	+
CDe/cDe	+	+	—	+	—	+
CDe/cde	+	+	—	+	+	+
Cde/Cde	+	—	—	—	+	+
Cde/CdE	+	—	+	—	+	+
Cde/cDE	+	+	+	+	+	+
Cde/cdE	+	—	+	+	+	+
Cde/cDe	+	+	—	+	+	+
Cde/cde	+	—	—	+	+	+
CdE/CdE	+	—	+	—	+	—
CdE/cDE	+	+	+	+	+	—
CdE/cdE	+	—	+	+	+	—
CdE/cDe	+	+	+	+	+	+
CdE/cde	+	—	+	+	+	+
cDE/cDE	—	+	+	+	—	—
cDE/cdE	—	+	+	+	+	—
cDE/cDe	—	+	+	+	—	+
cDE/cde	—	+	+	+	+	+
cdE/cdE	—	—	+	+	+	—
cdE/cDe	—	+	+	+	+	+
cdE/cde	—	—	+	+	+	+
cDe/cDe	—	+	—	+	—	+
cDe/cde	—	+	—	+	+	+
cde/cde	—	—	—	+	+	+

The symbols C, D, E, etc., are explained in the text.
The symbol / is used to separate the two chromosome formulas (Fishers)=genes (Wiener).

no proof negating Wiener's theory of multiple Rh-Hr allelomorphs has appeared. Familial studies[128] have so far shown that the genes of a parent are inherited as a unit by the offspring. The present tendency is to accept Wiener's theory of the mechanism of inheritance of the Rh-Hr genes.

Rhesus Antigens.- When first discovered, the Rh antigen was thought to be a single substance common to both the erythrocytes of the Macacus rhesus and those of Rh positive individuals.[78] Since then, the rhesus antigen has been found to be similar to the most antigenic of the Rh substances, D. There are indications that the rhesus antigen and human D are not identical. The investigations of Landsteiner and Wiener[78] and of Davidson and Tobarsky[28] demonstrated that there were differences in specificity of antisera produced in response to rhesus and human erythrocytes. Since the varieties of Rh and Hr antigens were not known at the time, no conclusions can be drawn as to the relationship of the rhesus Rh antigen and the human factor, D. Fisk and Foord[41] found that Rh antisera produced in guinea pigs in response to rhesus erythrocytes would differentiate among Rh +/- and Rh negative adults, but strongly clumps all infants' red cells. This observation was confirmed by Stratton.[128] Whether lack of specificity was caused by the source of the antigen or by the source of the antisera has not been indicated. No

studies have been published in which animal anti-human-Rh -/- erythrocyte sera have been tested against infants' cells.

Types of Human Rh-Hr Antigens.- The existence of the various Rh-Hr antigens has been demonstrated by the immune responses of individuals who have received red cells containing incompatible Rh factors either by transfusion or, in pregnant women, by passage of antigens of the fetal cells into the maternal circulation. The existence of these antigens has also been demonstrated by the reactions of red cells of the individual with known Rh-Hr antisera.

The first Rh antibodies were found by Landsteiner and Wiener[77] in rabbits immunized against rhesus red cells. These antibodies were compared by Wiener and Peters[138] with antibodies from 2 individuals transfused with blood known to be of the same group, but who showed transfusion reactions. The rabbit and human antibodies were found to give parallel results when tested against the red cells of various individuals. On further investigation the red cells of 85 per cent of the white population were found to be agglutinated by these antibodies. This antigen is now designated Rh_o(D). Clinically, an Rh positive person has the Rh_o(D) antigen, the most potent immunizing antigen of the Rh-Hr series, in his red cells. It accounts for approximately 98 per cent[120] of human sera containing Rh antibodies. Both agglutinins[78],

[81,126] and blocking antibodies[34,126] to this antigen have been demonstrated. Anti-D antibodies have been produced in response to transfusions, to stimulation by fetal red cells, and to artificial immunization. This antigen has slight dosage effect.[116] That is to say, there is little difference in strength of reaction with anti-D serum between the cells of homozygous individuals, DD, and those of heterozygous individuals, Dd.

Stratton[129] discovered a second allelomorph for the gene D by testing the cells of an individual CDe/cde with a number of strong anti-D sera. These cells gave variable reactions with the different sera. With some they agglutinated strongly, with some weakly, and with some no reaction was observed. This suggested the presence of another allel for the gene D. The former antigenic expression was weaker than that of the D gene. The D^u sera of Stratton were mixtures of anti-D and anti-D^u. No pure anti-D^u sera could be produced by absorption of the mixture with cells containing the D antigen. When Stratton, by injecting the cells CD^ue/cde, attempted to immunize two Rh negative individuals (one showing a mixture of anti-D and anti-D^u antibodies in his sera, the other none) there was no production of anti-D^u nor increase in titer of the same. However, anti-D^u was produced in an artificially immunized male inadvertently. In Van Loghem's[97]

attempt to produce a pure anti-E antibody, cells supposedly of cdE specificity were used. However, in testing for antibodies, both anti-D and anti-E were observed. On retesting the donor's cells, the latter were found to be of cD^uE. As suggested by Stratton this case indicated that typing with pure anti-D serum put a cDE individual into a cdE category. Whether there is any dosage effect could not be determined, for no individual who was homozygous for D^u was available.

Small d, the Hr antithetical allelomorph of the antigen D, was obtained by Diamond[18] and by Hill and Haberman.[65] The latter reported 2 antisera produced by iso-immunization of mothers. Both sera contained anti-d agglutinins with anti-c blocking antibodies. Whether Diamond's anti-d[37] was pure was not reported.

There are 5 allels for the C locus.[118] The first anti-C serum was reported by Levine, Burnham, Katzin and Vogel[85,86] in 1941. Three human sera were tested against a series of unselected bloods. One gave a 73 per cent incidence of agglutination. In the light of current knowledge it was a pure anti-C antibody. In the same year Wiener[139] reported a "70" per cent serum. Since then, there have been numerous instances of anti-C antibodies appearing in the sera of people transfused with incompatible blood and in those of iso-immu-

nized gravid females. Most anti-C sera are mixtures of anti-C and anti-D. If the anti-D antibodies are blocking antibodies, and if the anti-C are agglutinins, such sera may be used to type cells suspended in saline. If both are agglutinins, anti-D blocking antibodies can be added. These prevent the action of anti-D agglutinins, and the sera then can be used against cells in saline. The most desirable sera contain only anti-C antibodies. Van Loghem[97] attempted to produce a pure anti-C by immunization of a male volunteer. Such antibodies were obtained; agglutinins were present to a titer of 1:32, blocking antibodies to a titer of 1:128. However, anti-D^u antibodies were also produced. The C antigen has little dosage effect.

The second allel, C^w, is antigenic in blood transfusions, and produces hemolytic disease of the newborn.[80] Race, Mourant and Callender[116] found that about one half their anti-C sera actually contained a mixture of anti-C and anti-C^w, thus apparently explaining the discrepancies found in the behavior of different anti-C sera. Only blocking antibodies have been found, no agglutinins. There is a very definite dosage effect. The cells of homozygous individuals react more strongly than those of heterozygous individuals. As indicated by Race[119] the genetic relationship between C and C^w is clear, but not the antigenic relationship. C can stimulate the pro-

duction of both anti-C and anti-C^W. Naturally occurring mixtures of anti-C and anti-C^W cannot be split by absorbing with C or C^W cells. An artificial mixture made by adding pure anti-C to pure anti-C^W can be split by absorption. Unlike C, C^W has a strong dosage effect. The cells of a homozygous individual were clumped much more strongly by pure anti-C^W than those of a heterozygous person.

Evidence for the third allel,[116] C^u, is still incomplete.

The antithetical antigen, c, is the most antigenic of the Hr series. An antiserum was observed by Levine[90] which agglutinated all Rh negative cells and many Rh positive cells. Since it had an inverse relationship to the Rh antigen, he termed it Hr. Shortly afterwards Race and Taylor[121] obtained a more potent antiserum of the same specificity. Further reports of such antisera have been made, and anti-c antibodies have been produced in response to transfusions and transplacental iso-immunization. Since the cells of homozygous individuals react much more strongly than heterozygous, there is a dosage effect.

Evidence of a fifth allel, c^V, has been suggested by Race, Sanger and Lawler[116] but proof is lacking. According to the authors, c^V bridges the gap between C and c. When anti-C-c^V serum is absorbed by either c^Vc or Cc cells, all

agglutinins are removed. The use of the term c was indicated by 2 factors. One was that the cells gave a double dose effect with anti-c sera. The second was that c^V segregated with genes D and E. CDE is a rare chromosome in England, cDE a common one.

The Rh antigen E was discovered by Wiener[142] in 1943. This antiserum only reacted with 30 per cent of the population. Race and Taylor[111] obtained such antisera from 2 Rh positive (having antigen D) mothers in the same year. Anti-E sera, particularly in the pure form, are rare. Dick[38] reported a pure anti-E agglutinin in an Rh negative woman. Rice and Watson[120] found a pure anti-E antibody in an Rh positive woman. Pure anti-E agglutinins, followed by the appearance of blocking antibodies, were produced in an Rh negative male volunteer by Van Loghem.[97] There is little or no dosage effect.[116]

The antithetical gene e is rarely antigenic. The first anti-e was found by Mourant[103] in an Rh positive patient, CDE/cDE, who had received numerous transfusions. A strong dosage effect was noted. Cells from a homozygous individual reacted more strongly than those of a heterozygous individual to the anti-E serum.

Location of Rh-Hr Antigens.- Saliva has been investigated for its content of Rh antigen using the inhibition technic with known anti-Rh sera and known Rh positive red cells. Levine and Katzin[88] and Wiener and Forer[140] were unsuccessful but Boorman and Dodd[3] and Mohn and Witebsky[101] demonstrated Rh antigen in saliva. Of the 14 samples from Rh negative people, none inhibited the 2 anti-Rh sera tested. Of the 51 samples from Rh positive people, 27 showed slight inhibition and 24 showed none. Mohn and Witebsky examined 30 samples of saliva. In 2 or 3 instances, they had the impression that small amounts of Rh substances were present. Their conclusions were that the appearance of Rh substances in saliva could not be determined with certainty. If present, they were in such low concentrations as to make their demonstration difficult because of the viscosity of the saliva. Boorman and Dodd consider that the almost complete insolubility of these antigens in water is the probable explanation of the above finding.

Gastric juice has been tested for the presence of Rh antigens by Mohn and Witebsky,[101] who found Rh antigens in 12 of the 20 samples from Rh positive persons and none in 2 from Rh negative individuals. Seven of the 12 samples showed relatively large amounts of Rh substances. Rh substances in gastric juices may be specific, but the majority

of the positive gastric juices inhibited agglutination of both Rh_1 and Rh_2 cells.

Seminal fluid and sperm cells, when examined by Levine and Katzin,[88] were not found to contain Rh substances.

Amniotic fluid contains Rh substances as indicated by the investigation of Witebsky and Mohn[161] and Mohn and Witebsky.[101] Of 100 samples of amniotic fluid, 71 of 86 samples from Rh positive and none from 14 Rh negative infants were positive. Thus, among Rh positive infants about 4 out of 5 are secretors. The presence of Rh substances in the amniotic fluid is dependent on the infant's not the mother's blood group. The secretion of Rh substances into amniotic fluid seems to be independent of the secretion of group specific substances. There were some instances of Rh_1 and Rh_2 substances, but most of the positive amniotic fluids reacted with both Rh_1 and Rh_2 cells. Three Rh positive erythroblastotic infants were non-secretors. Mohn and Witebsky found that more definite differentiation between secretors and non-secretors could be obtained by purification and concentration of the amniotic fluid. This was accomplished by dialysis of the fluid, removal of precipitate, lyophilization, and resolution of the dried material in 1/10 the original volume of saline.

Various tissues have been examined for Rh antigens by Boorman and Dodd.[3] Most of the analyses were made on liver, kidney, and spleen, and in some cases on heart, suprarenals, pancreas, brain, and salivary glands. Aqueous suspensions of ground-up tissues and aqueous extracts of tissues were tested by the inhibition technic. Aqueous extracts of tissues were entirely negative, but tissue suspensions were positive. They found that the Rh substances in tissues are relatively insoluble in water but very soluble in alcohol, and that there is considerably more Rh substance in the body tissues than in the body fluids. These findings have yet to be confirmed by other workers.

Chemistry of Rh-Hr Antigens.- Using physical methods, Belkin and Wiener[1] were able to concentrate Rh antigens 8 to 32 times, as determined by the inhibition technic. Their method was to combine Sharples centrifugation with running the material through the Travis colloid mill. The end product consisted of uniform minute particles.

Calvin et al[12] obtained stroma by Sharples centrifugation of hemolyzed human erythrocytes. In testing Rh positive and Rh negative stroma for specificity by the inspection method, they found that only stroma from Rh positive cells gave inhibition. The stroma were also tested for

their sensitivity to heat under varying atmospheric conditions. If exposed to 56 C for 5 minutes in saline, inhibiting ability of the stroma was destroyed. If exposed to the same temperature for the same length of time, but in air or vacuum without moisture, there was no decrease in inhibitory activity.

These authors were able to separate the stroma into 2 fractions by adjusting the pH. One fraction, called elinin, inhibited Rh antisera. The other, stromatin, had no inhibitory power. Elinin contained 40-50 per cent alcohol:ether extractable lipids; stroma contained 20 per cent. The Rh substance in the ether soluble fraction is more stable to heat than the Rh substance in stroma, resisting 56 C for one hour. When the ether-soluble substance is recombined with protein, the material is thermolabile again.

Carter,[16] by extracting human erythrocytes with alcohol and ether, obtained a lipid Rh inhibitor substance which she called the Rh hapten. It is essentially the same as that described by Calvin et al. Two methods for assaying the Rh substance were used. One tested the ability of the substance to fix complement in the presence of potent Rh antisera. The second tested its capacity to inhibit potent Rh antisera.

That this lipid substance is a hapten is indicated by its non-antigenicity when injected into 96 guinea pigs. How-

ever, when it was combined with a protein carrier, in this instance egg albumen, 5 of 24 inoculated guinea pigs produced antibodies to group O, Rh positive human red cells. Since no further information was given concerning the number of Rh positive cell suspensions tested or reactions with Rh negative red cells, it is not possible to determine whether Rh antibodies actually were produced.

In both immunized guinea pigs and iso-immunized humans, the *in vivo* neutralization of Rh antibodies was demonstrated by the fall in antibody titer after injection of the lipid.[17]

Rh Antisera

Human.- There are 5 main methods of actively immunizing humans against the Rh-Hr antigens. (1) The unavoidable response of reactive gravid females to the red cells of the fetus which are incompatible for the Rh-Hr factor. (2) The inadvertent transfusion of an individual with such incompatible red cells. (3) The hyperimmunization of iso-immunized individuals. (4) The active immunization of human volunteers. (5) Heterospecific immunization along with specific immunization of volunteers.

Since 1939 numerous instances of Rh sensitization may be found in the literature. Both agglutinins and blocking

antibodies have been observed. Such human antisera have provided the material for most of the studies on Rh antigens and antibodies.

Hill, Haberman and Orozco[64] injected 2 mothers of erythroblastotic infants intravenously with small doses of Rh positive cells. In 1 woman injections were started 6 days after delivery. At that time only blocking antibodies with a titer of 1:4 were present. The titer of agglutinins after immunization was 1:2,048, that of blocking antibodies 1:8. The response was probably a combination of the usual postpartum rise of Rh antibodies and immunization. The second case showed a postpartum titer of 1:2,048. Eighteen months later the titer had fallen to 1:64. After hyperimmunization the titer had mounted to 1:2,560 by the twelfth day.

Diamond, Wiener and van Loghem have actively immunized male volunteers. Diamond[36] was able to immunize 16 of 35 men. He gave them an initial intravenous injection of 50-500 ml of blood followed by 0.05 to 0.3 ml of blood 3 times a week for 3 weeks. Wiener and Sonn-Gordon, who immunized 9 men by 2 injections of 2 ml of blood 4 months apart, obtained antibody production in 2 but in lower titers than those obtained by Diamond. Male volunteers were then injected with small amounts of Rh_1 and Rh_2 red cells at 6-week

intervals over the period of a year. Five of 6 persons produced useful Rh antisera. Wiener observed that when Rh_2 blood was injected, anti-D agglutinins were the first to appear, then anti-D blocking antibodies, and finally anti-E agglutinins. When Rh_1 cells were used, the observations were similar. Anti-C were the last antibodies produced. Of 17 men immunized by Van Loghem,[97,98] who used frequent intravenous injections of small amounts of blood (Cde and cdE), 4 produced Rh antibodies. Two produced anti-C sera in response to Cde cells. In 1 the titer of the serum in saline was 1:8 and 1:16 in albumin, and that of the other was 1:32 in saline and 1:512 in albumin. Another produced low titers of anti-D, 1:2 in saline and 1:4 in albumin. The anti-E serum contained antibodies that would react only in albumin to a titer of 1:64. One of 3 nuns injected by Van Loghem with C^W antigen produced a pure C^W blocking antibody with a titer of 1:8 after 20 injections.

Van Loghem observed 2 particularly interesting facts. One was a fall in titer of the anti-E blocking antibody despite continued injections of the antigen and no response on hetero-immunization. This would indicate that an overdose of the Rh antigen is possible in artificial immunization of humans. The second observation was the exclusive finding of blocking antibodies without the appearance of

agglutinins in 1 person. In the other 3 antisera, agglutinins were followed by blocking antibodies.

The influence of hetero-immunization on the production of Rh antibodies was studied by Van Loghem[98] on 14 male volunteers who had previously received from 15 to 42 injections of Rh antigen without producing Rh antibodies active in either saline or albumin. Three of the 14 produced Rh antibodies after injections of triplovaccine (TAB) and tetravaccine (TABC) along with injections of the Rh antigen. It is interesting that the 3 showed clinical signs of sickness and rise of temperature in response to the vaccine.

The observation that heterospecific immunization stimulated the production of Rh antibodies is important. Wiener[145] has suggested the use of heterospecific immunization with typhoid vaccine to lower the Rh antibody production in gravid females on the assumption that a more potent antigen such as typhoid vaccine would compete with the weak Rh antigens at the sites of antibody production. Levine[96] stated that such injections might well stimulate further response rather than act as a diversion. The experiment of Van Loghem corroborates this point. However, the data of Van Loghem on the effect of hetero-immunization would have been more convincing if the Coombs test had also been carried out with the sera of the group that showed no antibodies by other

technics.

Transmission of fetal cells into the mother.- Wiener[153] considers that leakage of fetal red cells into the maternal circulation rarely occurs in the course of pregnancy, but at the time of labor and at parturition, when there is disturbance of the placenta. This is suggested by the fact that congenital hemolytic disease rarely occurs with the first-born unless there is a history of previous transfusion.

Levine[96] believes that iso-immunization is probably initiated toward the latter half of pregnancy when the blood vessels in the villi gradually approach the maternal sinuses and are in intimate contact over a large surface. The facts that support Levine's theory are: A single injection of Rh positive cells into an Rh negative individual rarely causes demonstrable antibody production. The degree of sensitivity in transfused individuals rarely approaches that of Rh negative mothers of erythroblastotic infants unless multiple transfusions are given. This would indicate that the Rh negative mother has had the optimal opportunity for immunization, which is continuous exposure to the antigen over a long period of time. Another fact that points to a continuous antigenic stimulus is the increase in antibody titer that is observed during pregnancies following the sensitizing pregnancy.

Genetic predisposition toward antibody production.-Haldane[60] has postulated that a gene exists in man determining placental permeability to formed elements. However, if we assume that minute quantities of fetal blood pass the placental barrier, as Levine's theory indicates, this must occur in every pregnancy.

Wiener[153] has explained the varying responses to the Rh antigens on the basis of a particular gene which he calls K. A homozygous individual who carries KK will be very susceptible and produce a large number of antibodies in response to a mild stimulation. The individual who is homozygous for kk will not react at all to Rh antigens by antibody production. The heterozygous individual Kk will produce Rh antibodies if strongly stimulated. There is no factual basis for this gene.

Levine[92] has suggested that individuals vary in their ability to produce Rh antibodies and that this ability is a general expression of the person's genetic constitution.

Preparation of anti-Rh sera.- The antibodies used for typing red cells for the Rh factor are primarily agglutinins. When unavailable, blocking antibodies are used. As indicated above, the main sources of Rh antibodies are from sensitized pregnant women and transfused males and females. Increasing use is being made of volunteers. Additional sources

of antibodies were made available by the absorption of blocking antibodies and by concentration of sera too weak for use in their original form. According to the standards of the National Institute of Health[133] an Rh antiserum must have a minimal titer of 1:32 units. If diluted, the protein content must not be reduced below 25 per cent of normal.

The normal anti-A and anti-B iso-agglutinins must be removed before human sera are specific for Rh. This procedure is accomplished either by absorption of sera of individuals of Group A, B, and O (individuals of Group AB contain no iso-agglutinins in their sera) with Group A and B Rh negative red cells or by the addition of group specific substances A and B. The A substance is derived from the gastric mucosa of hogs, AB substances from the gastric mucosa of horses.[164] The latter method is preferable since there is no nonspecific decrease in titer of the Rh antibodies. It is necessary to check anti-Rh sera periodically for loss of titer and specificity. Cappell and McFarlane[13] found that anti-Rh sera may develop weak anti-A and anti-B iso-agglutinins on standing.

Advantages and Disadvantages of Human Anti-Rh Sera.- One of the most important advantages of human Rh-Hr antisera is their specificity. So far no other animal has been able to produce antisera to the Rh subtypes or to variations with-

in those subtypes. Good human anti-Rh sera are more potent than the best animal antisera. This advantage makes the Rh typing of red cells more accurate. With human Rh antisera it is possible to differentiate Rh positive from Rh negative infants, whereas the animal anti-rhesus sera agglutinate all infants' cells strongly.

On the other hand there are certain disadvantages. Many human Rh antisera contain more than 1 antibody, and the presence of blocking antibodies may mask the action of the agglutinins if both are of the same specificity. Many human Rh antisera contain antibodies to more than 1 antigen. If these mixtures contain 1 antibody in the form of agglutinins and another in the form of blocking antibodies, the latter may be absorbed out, but the presence of 2 separate agglutinins is a more difficult situation to deal with. The source of Rh antisera is not constant, for the titer of donors is maintained for a variable length of time. Except for anti-D sera, good Rh antisera cannot be produced in large quantities or at will.

Animal.- Since the original discovery of an anti-Rh agglutinin in anti-rhesus rabbit's serum by Landsteiner and Wiener in 1940,[77] numerous workers have tried to produce anti-Rh sera in animals with varying success. All of the

published work has been done with guinea pigs and rabbits, horses[6] and goats[7] being immunized without success. Most workers have used erythrocytes from Macacus rhesus as the antigenic stimulus, some have employed human Group O, MN Rh positive erythrocytes, one worker human stroma, and another a lipid extract from human Rh positive erythrocytes.

When Landsteiner and Wiener were unsuccessful in obtaining a similar agglutinin by the injection of more rabbits with rhesus red cells, they were able to produce Rh agglutinins by intraabdominal injection into an unspecified number of guinea pigs.[78] The sera was rendered specific for Rh either by diluting to a point where it would not give any reaction with human Rh negative red blood cells but would clearly agglutinate Rh positive cells, or by absorbing the sera diluted 1:4 with saline with sufficient human Rh negative red cells to remove the reaction with the Rh negative cells. Landsteiner and Wiener defined a diagnostic serum as one which, when serially diluted by halves, and tested against known positive and negative erythrocytes, showed negative reactions in 3 or more successive dilutions with the negative cells, and positive reactions in those dilutions with the positive cells. Using this criterion, these authors found that one or more of 10 guinea pigs produced diagnostic sera. They checked sera from different

sources to see how they compared. Forty-two bloods were tested with the original rabbit's serum and with human sera from post-transfusion cases. Parallel reactions were obtained. The sera of 3 guinea pigs were checked with 110 random bloods with similar results. Likewise, 159 bloods gave parallel reactions when tested with a guinea pig anti-rhesus serum and a human serum from the mother of a child with erythroblastosis. There were 3 cases of discrepancies and 3 that gave doubtful reactions with the guinea pig's serum.

In 1942 Landsteiner and Wiener[79] modified their method for immunizing guinea pigs against rhesus erythrocytes. The longer immunization gave a higher proportion of usable serum and made it imperative to use the absorption technic to eliminate agglutinins other than anti-Rh.

Davidson and Toharsky[28] inoculated guinea pigs intra-abdominally with washed rhesus erythrocytes. The guinea pigs' sera were used on the basis of differential titration between Rh positive and Rh negative cells, no attempt being made to absorb out nonspecific agglutinins. Of the 12 guinea pigs injected, 5 produced agglutinins that could differentiate between Rh positive and Rh negative red cells, but only 1 was strong enough to be used diagnostically. Of the

11 rabbits injected none produced diagnostically usable antisera. There was some slight difference in titer between reactions with Rh positive and Rh negative bloods. The titers with Rh positive cells ranged from 1:3,500 to 1:7,000 and those with Rh negative cells 1:1,500 to 1:3,500. Absorption with Rh negative cells in an attempt to remove the agglutinins for the Rh negative cells, inhibition with Witebsky's A and B substances[160] and with A and B saliva[140] all failed to produce a usable Rh antiserum. Comparative tests performed on 61 human bloods with 1 guinea pig and 3 human Rh antisera revealed 50 parallel results and 11 showed discrepancies. When 25 human bloods were tested with 2 rabbit's antisera and 3 human antisera, 18 were in agreement and 7 showed differences.

Fisk and Foord[41] also produced Rh agglutinins by injecting guinea pigs with rhesus red cells. The preparation and injection schedule of the antigen, bleeding schedule, and tests for agglutinins were similar to those used by Landsteiner and Wiener.[78] They found that absorbing the serum diluted 1:10 with 1/2 volume of Rh negative red cells gave more definite differentiation between Rh positive and Rh negative cells. The number of animals injected was not given, but the authors stated that the sera varied considerably in suitability, had weak antibody production, and failed to distinguish between Rh positive and Rh negative bloods.

Of 927 white adults chosen at random 15 per cent were Rh negative and 85 per cent Rh positive, in agreement with the observations of Landsteiner and Wiener. Using 4 potent human sera from mothers with erythroblastotic infants random samples of adult bloods revealed similar proportions of Rh positive and Rh negative bloods. Simultaneous tests with guinea pigs' anti-rhesus sera and 2 human anti-Rh sera gave the following results: In testing 197 bloods with 1 human serum and guinea pig's serum, 192 gave parallel results and 5 were positive with the human antiserum and negative with the guinea pig's antiserum. With a second human serum, 79 of 83 bloods gave parallel reactions and 4 showed discrepancies.

All of 312 cord bloods of infants gave strongly positive reactions with the guinea pig's anti-Rh serum. Tests with human anti-Rh sera differentiated Rh positive and Rh negative infant cells. Blood collected from 9 infants 10 days after birth gave results similar to those with cord bloods.

Two years later Fisk and Foord[42] in comparing the sensitivity of guinea pig's anti-Rh serum with those obtained from 8 mothers of erythroblastotic infants, found that 4 of the human sera gave parallel reactions to the guinea pigs' antisera and 4 gave fewer positive reactions. One of the

human antisera was found to react with only 70 per cent of the series of adult blood samples. Absorption with Rh negative cells was used again, for this method resulted in better differentiation of Rh positive and Rh negative cells.

In 1943 Gallagher and Jones[54] injected guinea pigs and rabbits with rhesus red cells. The preparation of the antigen and the injection schedule for the guinea pigs were similar to the methods of Landsteiner and Wiener.[78] Bleedings were taken 1 and 2 weeks after the last injection. To absorb nonspecific agglutinins, 1 volume of serum was diluted with 4 volumes of salines and 1 volume of Rh negative cells was added. In some cases 2 and 3 absorptions were necessary. If the initial antibody content was low, absorption would remove all activity. Half the guinea pigs showed agglutinins that distinguished between Rh positive and Rh negative cells, and about 1 out of 5 produced diagnostic serum. No Rh antibodies were obtained from immunization of rabbits. The anti-rhesus guinea pigs' sera were compared with 2 sera from mothers with erythroblastotic infants. Of 156 bloods tested with the guinea pig's serum 83 per cent gave positive reactions. Of 118 bloods tested with 1 of the human sera 85 per cent gave positive reactions. When the anti-rhesus and the above human sera were tested simultaneously with a second human serum, the first two gave parallel reactions, whereas

the second human serum only reacted with 54 per cent of the bloods.

McIvor and Lucia[104] tested 4 different injection schedules immunizing guinea pigs with rhesus erythrocytes. The absorption technic was used to produce specific Rh antisera. Sera giving clear cut Rh positive and Rh negative sediments were called diagnostically usable. The results were: 3 of the 18 animals given the first injection schedule produced usable sera; of the 5 guinea pigs on the second schedule 2 produced high titered sera and 1 fair; of the 7 survivors on schedule 3, 3 produced high titered sera; 5 of the 9 animals on schedule 4 produced good sera.

Ecker, MacFarlane and Laipply[40] injected an unspecified number of guinea pigs with rhesus erythrocytes, some being injected according to Landsteiner and Wiener's[78] procedure and some subcutaneously. Sera from 2 animals were pooled, inactivated at 56 C for 30 minutes, and absorbed with Group A and Group B Rh negative cells (details were not reported). Human cells were called Rh positive or Rh negative on the basis of differential titrations. The criteria for differentiating Rh positive from Rh negative cells were not clear. Of 125 bloods of known types tested with guinea pig's anti-rhesus serum, 19 or 15 per cent were Rh negative. Rh agglutinins were completely removed from guinea pig's antiserum

by absorption with human Rh positive red cells.

Guinea pigs and rabbits were injected with rhesus red cells by Homburger,[67] who studied inhibition of production of Rh antibodies. The experimental group was injected with sodium salicylate to depress antibody formation. When the sera were collected, and whether or not they were made specific for Rh antibodies was not indicated. The sera of guinea pigs were examined for agglutinins using Landsteiner and Wiener's[78] method and the sera of rabbits were tested by Diamond's method[33] using human albumin. Of 40 guinea pigs originally injected, 31 survived. Of 20 rabbits, 8 survived. In reporting the results both rabbits and guinea pigs were combined. Twenty-one animals were in the control group. Of these 15 showed anti-Rh agglutinins (the criteria for defining Rh agglutinins were not reported). The authors stated that the rabbit's antibodies were stronger. However, different methods of testing were used. Of the 18 animals receiving sodium salicylate only 2 showed anti-Rh agglutinins.

Scherer[122] injected rabbits with rhesus red cells in an attempt to inhibit the production of Rh antibodies. As inhibiting agents, he tested ethylene disulfonate, sodium salicylate, pyrabenzamine, and A and B blood group substances. There was little difference in antibody production when tested against red cells suspended in saline and against red

cells suspended in Group AB serum. If the agglutinins were Rh agglutinins (this cannot be determined from the paper since no Rh negative cell suspensions and only one Rh positive cell suspension were used) such findings would indicate that only agglutinins and no blocking antibodies were present. None of the substances tested inhibited agglutinin production. (If these were Rh agglutinins, and Homburger's antibodies also were Rh agglutinins, these observations would contradict Homburger[67]). Another procedure which leaves the nature of agglutinins in doubt was the use of a one-step absorption employing 1 part serum and 5 parts of packed cells. The Rh agglutinins are quite delicate, and one would think that such a procedure would absorb out any Rh agglutinins nonspecifically.

A few experiments have been carried out in animals using human Rh positive erythrocytes as the antigen. In 1939 Levine and Stetson[81] injected human cells that could be agglutinated by human atypical agglutinins into rabbits in an attempt to reproduce the antibodies in animals. After absorption with human Rh negative cells, no agglutinins remained. Again in 1941 Levine and Polayes gave 4 guinea pigs and 4 rabbits a series of injections with Group O, MN, Rh positive red cells. No Rh agglutinins were found.

Gallagher and Jones[55] published a note in 1943. Guinea pigs were inoculated with Group O, MN, Rh positive red cells over a 3-month period. One or 2 weeks after the last injection of antigen, the animals were bled and the sera absorbed with Group O, MN, Rh negative cells using the method described in their paper on rhesus cells as antigen. They stated that such sera distinguished sharply between known Rh positive and Rh negative cells of all groups. No further data were given. No indication was given as to how these sera would react with infants' cells.

In 1945 Carter[14] injected 6 guinea pigs with human Group O, MN, Rh positive erythrocytes. According to the author sera previously absorbed with Rh negative cells showed a clear cut reaction with positive, and no reaction with negative cells. Simultaneous tests were performed on 500 bloods with standard anti-D typing serum and guinea pig's serum, with parallel results.

In a later report on a substance which inhibits anti-Rh sera,[16] 24 guinea pigs were injected with a 10 per cent suspension of Group O, MN, Rh positive cells intraabdominally. The author stated that each control animal produced agglutinins to human Group O Rh positive cells up to titers of 1:160 and above. No mention was made of a method for making these sera specific for the Rh antibodies, nor of any reactions

with Rh negative cells.

Gallagher and Pillischer[56] used stroma prepared from human Rh positive cells as the antigen. With absorbed sera it was possible to distinguish between Rh positive and Rh negative red cells. No mention was made of the number of animals involved, the number producing good anti-Rh agglutinins, the actual injection schedule, nor the criteria that a good anti-Rh serum would have to meet.

Erythrocytic Extracts.- Carter[16] in 1947 published a preliminary report on a substance which inhibited anti-Rh human serum. The preparation of the material is given in the section on chemical studies under Rh antigens. None of the 24 guinea pigs and 2 rabbits produced antibodies to human red blood cells. When equal volumes of 10 per cent lipid suspensions in 50 per cent alcohol and 10 per cent egg albumen in saline was injected intraabdominally into another series of guinea pigs, 5 of 24 developed agglutinins for Group O, Rh positive red cells. The author gave no data dealing with removal of nonspecific agglutinins, if any, or indicating the reactions of the antisera with Group O, Rh negative red cells.

In a later paper[17] they stated that the injection of this material into guinea pigs which had anti-Rh antibodies

in their blood stream caused a reduction in titer of those antibodies.

Advantages and Disadvantages of Animal Rh Antisera.- The main advantage is the ability to produce sera at will. Since animals, as far as is known, produce only agglutinins not blocking antibodies, agglutinins would not be masked. With careful determination of the specific Rh antigens and the absorption of nonspecific agglutinins, it might be possible to produce "pure" Rh antisera of the subtypes.

There are several disadvantages to the use of animal sera. Approximately one half of the animals injected with Rh antigens do not produce Rh agglutinins. Only animal antisera with the specificity of anti-D have been observed so far. No animal antisera against the other 2 Rh antigens, or to the 3 Hr antigens, have been noted. Anti-rhesus antisera strongly agglutinate all infants' cells and only differentiate between Rh positive and Rh negative adult cells. The cells of infants have never been tested against anti-human-red-cell antisera of animals.

Types of Antibodies.- The presence of Rh antibodies is evidenced by their activity when brought into contact with their specific antigens under specified environmental conditions. There are two known categories of Rh antibodies,

agglutinins and blocking antibodies, and a third, (postulated kind of antibody), "cryptagglutinoids" or "developing" antibodies.

Rh agglutinins are specifically modified gamm globulins[36, 151] that are found in the serum of immunized humans and animals, attached to the red cells of sensitized infants,[15,58,137] and present in the milk of postpartum women who have agglutinins in their plasma.[158,159] The Rh agglutinins coat the Rh positive red cells and cause them to agglutinate when suspended in saline or in various colloidal solutions. Titrations of these agglutinins can be made in saline or in the various colloidal solutions. Other terms used to denote Rh agglutinins are: Wiener's "bivalent",[151] Diamond's "thermolabile" or "early" or "immature" antibody,[33] Coomb's "complete" antibody.[23]

Rh blocking antibodies are specifically modified beta globulins[36] found in the serum of immunized humans and attached to the red cells of sensitized infants.[137] Whether they are present in the breast milk of women with blocking antibodies in their plasma has not been investigated. There is no evidence for their presence in animal Rh antisera. These antibodies coat the red cells and saturate the Rh combining sites of the cells.[66] They produce no agglutination in the presence of saline, but cause clumping when the cells are

suspended in colloidal solutions. Their attachment to the red cells when suspended in saline is shown by further addition of known Rh agglutinins to the test cells.[143] The inhibition of agglutination indicates that the antigenic sites on the red cell have been blocked. A more delicate test consists of adding an anti-antibody, e.g., rabbit anti-human-serum antibody, to the sensitized cells.[23] Clumping will occur if the blocking antibodies are present. If in the test a colloidal solution, such as serum,[146] plasma,[151] albumin,[33] gelatin,[43] glue,[33,92] and others, is substituted for saline, blocking antibodies will cause direct clumping of the red cells.

Other terms used to denote Rh blocking antibodies are: Wiener's[151] "univalent" or "glutinins", Diamond's[33] "thermostable", "late", or "mature", Coomb's[23] "incomplete" antibody.

"Developing" antibodies or "cryptagglutinoids" or third order antibody are the terms suggested by Hill, Haberman and Jones[66] for a postulated third order antibody. This antibody coats Rh positive red cells suspended in saline, but does not saturate the sites of antigen on the red cell. They are only demonstrated by the Coomb's or "developing" test.

Technical Methods.-The original test for the presence of Rh antibodies was the demonstration of agglutination.

Various modifications have been made in the technic of Landsteiner and Wiener, the most important being the incubation of the tests at 37 C and the introduction of gentle centrifuging before reading.[152]

Numerous workers found puzzling results when testing for the causes of intra-group transfusion reactions and of cases of erythroblastosis fetalis that were similar in every way to those due to Rh incompatibility except that no Rh agglutinins could be demonstrated in the maternal circulation. The explanation for such phenomena became apparent in 1944 when both Wiener and Race demonstrated the Rh blocking antibody. In testing the sera of patients reacting to intra-group transfusion reactions or those of mothers of erythroblastotic infants for Rh sensitization, Wiener[143] found that by the usual agglutination test, no antibodies could be demonstrated. However, when a drop of known active anti-Rh serum was added to the known cell-test serum mixture, the Rh positive red cells were not agglutinated, indicating that a reaction had occurred at the sites of Rh antigen on the red cells with antibodies of the unknown serum. The reaction was found to be specific for Rh positive red cells, occurring only with sera from Rh negative people sensitized to the Rh factor.

Race[114] discovered the blocking antibody when studying mixtures of different types of human anti-Rh sera. The

initial observations that led to this discovery were as follows: Anti-D serum reacts with CDe and cDE cells. Anti-CDe serum agglutinates CDe cells but not cDE cells. If cDE cells were added to a mixture of the two sera, no agglutination occurred, contrary to the reaction that should have been observed due to the presence of the anti-D serum. Similar findings were found without mixing the sera. When cDE cells were suspended in anti-C serum without agglutinating, then separated, washed, and resuspended in saline, these cells could no longer react with anti-D serum. CDe cells could also be coated with this incomplete antibody. Agglutination of the coated cells did not occur when the pH and salt concentrations were varied. When a mixture of complete and incomplete anti-D antibodies were titrated, the titer was the same as when the serum with the complete antibodies was titrated except that the strength of reactions with the former was much weaker. This observation indicated that the incomplete antibodies reacted more rapidly with the antigenic sites of the red cells than the complete antibodies.

Diamond and Abelson[34] found an Rh inhibitor substance in anti-Rh sera that inhibited anti-Rh agglutinins. This inhibitor substance is equivalent to Wiener's blocking, and Race's incomplete antibody. In addition to anti-D blocking antibodies, anti-E and anti-c blocking antibodies were also

demonstrated. These workers showed that the degree of inhibition of agglutinins by blocking antibodies depended upon the relative amounts of these two antibodies present in a serum or mixture of sera. In an *in vivo* experiment they confirmed the finding that anti-Rh agglutinins may be completely masked by the inhibitor substance. Pooled serum that showed no agglutinins was injected intravenously into a CDe/cde person. Afterwards tests of the individual's blood showed free anti-D agglutinins in the serum, and some of the cells were inagglutinable, indicating that they were bound by the inhibitor substance. Similar results were obtained *in vitro* with the same serum and cells. When one part of cells was added to one or more parts of serum, no agglutination occurred. As the cell concentration was increased, agglutination became apparent. These findings led the authors to test the sera of sensitized women that showed no anti-Rh agglutinins with an excess of cells. Agglutination of Rh positive cells occurred.

Diamond and Abelson's[35] observation that anti-Rh agglutinins masked by an inhibiting substance in a serum could be demonstrated if tested against a higher concentration of Rh positive cells led to the development of the slide test. According to the authors the mechanism depends on the high cell concentration which leaves sufficient unblocked sites

on the red cells for action of the agglutinins. However, Wiener[168] has demonstrated that Diamond and Abelson's slide test was positive when only blocking antibodies and no agglutinins were present in the serum, thus ruling out the latter authors' idea that the agglutinins were unmasked. The authors stated that the method could not be used for Rh typing. Wiener pointed out that their slide test could be used for typing if antisera containing separate or pure antibodies were used. For instance, a pure anti-C, not a mixture of anti-C agglutinins and anti-D blocking antibodies, would have to be used, for this test permits both agglutinins and blocking antibodies to act. Other authors were unable to repeat the slide test using saline suspensions of 40-50 per cent washed erythrocytes. Also Diamond and Abelson[32] were unable to repeat their original results using cells suspended in saline. Wiener[148] and Levine and Waller[95] demonstrated that the determining factor was the use of serum as the suspending medium rather than saline.

A second slide test, recommended by Simmons[123], employs a 5 per cent red cell suspension in Rous-Turner solution.

As indicated above, blocking antibodies can be demonstrated directly by the test tube method when various media are substituted for saline both for suspending the red cells and for diluting the sera. Wiener[148] in 1945 substituted

Group AB or compatible human serum or oxalated plasma for saline, and called the procedure the "conglutination" test. Since the test worked equally well with fresh and with heated serum, and with plasma inactivated for 1/2 hour at 60 C, complement was not involved. Since slight dilution of the serum or plasma with saline weakened or eliminated the reaction the cells were not washed with saline. Later, Wiener advised washing the cells once with saline, then suspending them in the serum or plasma. Levine and Waller[95] obtained the same finding using serum as the suspension medium. Wiener's[148] routine procedure was to test the sera first for agglutinins. If negative, the tube was centrifuged, the supernate removed, and replaced by a drop of compatible serum or plasma. The tube was then reshaken, incubated at 37 C for one hour, and read.

Boorman, Dodd and Morgan[5] in 1945 also reported on the enhancing properties of compatible human sera on the agglutination of Rh positive red cells by Rh antibodies, but did not record the existence of previously unrecognized antibodies. They found that the plasma or serum of different individuals varied in their ability to enhance agglutination. In 1947 Wiener and Hurst[155] substituted a combination of oxalated plasma and 25 per cent human albumin in a proportion of 4 to 1. They found that the addition

of albumin to plasma increased the sensitivity of the reaction. Wiener[153] postulated that the mechanism of the test depended upon the presence of X protein, a molecular complex of albumin, globulin and phospholipid, in the serum or plasma. However, some other mechanism must function since colloidal substances work equally well.

There are conflicting reports concerning the use of umbilical cord serum as a suspension medium for the red cells and a diluent for sera in testing for the presence of Rh blocking antibodies. As seen below, the observations of various workers indicate that this discrepancy is due to individual variation among cord sera. Lubinski[99] stated that cord sera could not be used to demonstrate the incomplete Rh antibody. On the other hand, Levine[92] stated that human cord serum was not much less sensitive than pooled male serum as a suspension medium, and that certain cord sera are preferable to selected male sera.

Gurevitch, Polishuk and Hermoni[57] studied the activating effect of sera from infants of different age groups, and normal, pregnant, and diseased adults, on Rh blocking antibodies when substituted for saline. The blocking antibodies were obtained from two women who gave birth to erythroblastotic infants, one having an agglutinin titer of 1:8 and a blocking titer of 1:64, and the other an agglutinin

titer of 1:128 and a blocking titer of 1:256. It is doubtful that blocking antibodies actually were present since a 1 tube difference is well within the range of error of the test. The results when tested against the first serum were as follows: Of the 41 cord sera, 38 were weak and 3 activated the blocking antibodies like mature sera. Of 11 sera from infants ranging from 1-6 months of age, 10 were weak and 1 was mature. Of the 10 sera from infants ranging from 6-18 months of age, 1 was weak and 9 were mature. The sera from the normal adults, pregnant women, and adults with hepatic diseases were fully active. The authors concluded that the enhancing properties of mature human sera were not influenced by pregnancy or by cirrhosis or hepatitis of the liver. This property was not found in cord blood or in infants' blood up to 6 months of age.

In 1947 Witebsky, Rubin and Blum[163] found that the enhancing properties of fetal and infant sera on Rh blocking antibodies varied with the Rh serum and with the cord serum.

Varying activity among umbilical cord sera was demonstrated with a serum containing only blocking antibodies to a titer of 1:320 from a mother of an erythroblastotic infant. Three adult human sera, one of Group O, one of Group A, and one of Group B, and three cord sera were used both to dilute the serum and to suspend the Group O, Rh positive

test cells. The adult sera gave a blocking titer of 1:320. One cord serum gave a titer of 1:5, a second 1:20, and the third 1:160. There is also variation among anti-Rh sera in their ability to be activated by cord sera,and the capacity of cord serum to activate the blocking antibody also varies with the Rh antiserum tested. The serum of a mother of an erythroblastotic infant was compared with a commercial anti-Rh serum, which the authors assumed was produced by immunization of Rh negative volunteers with Rh positive cells. Reactions with the maternal serum produced a titer of 1:40 with the 3 normal adult sera, and no agglutination with the 3 normal cord sera. When the same normal adult and normal cord sera were used with the commercial Rh antiserum, all 6 showed a titer of 1:40. The authors suggest that these differences may depend on the titer of anti-Rh antibodies in the different sera and on the method of immunization. The activating capacity of the sera of premature infants was tested against a commercial anti-Rh blocking serum. When activated by Group O, A and B normal adult sera, a titer of 1:80-1:160 was observed. Of the premature sera, 1 six-month premature serum activated the blocking serum to a titer of 1:5, 2 eight-month 1:5 and 1:10, 1 two-week premature 1:20, 1 one-week premature 1:10. Of 3 full term, 2 activated the Rh antiserum to a titer of 1:40, 1 to 1:80. It can be seen that part of the activa-

ting capacity of sera of premature infants depends upon the age of the fetus. "Obviously a maturation factor plays a definite role in the appearance of the characteristics responsible for the activation of the incomplete Rh antibody--"

The effect of maturation in post-natal life was studied in (a) cord serum, (b) serum from venous blood 24 hours after birth, and (c) serum from venous blood 48 hours after birth. The anti-Rh blocking serum with a titer of 1:320 was from a mother of an erythroblastotic infant. None of the 3 samples of 1 infant showed any activating capacity; only the 48-hour sample from a second infant, and both 24 and 48 hour samples from a third infant activated the blocking antibody. From the testing of premature, cord, 24 and 48-hour serum samples, it is apparent that the activating power of serum depends upon a maturation factor. However, because of individual variation, it would be impossible to predict the activating power of a fetus' or infant's serum at any particular time.

There is a difference between adult and cord sera in total protein, albumin and globulin. On the average cord sera contained about 1 g per cent less of total protein and 1/2 g per cent less of albumin and globulin than in the average adult serum. However, some of the cord sera which had poor capacity to activate Rh blocking antibodies had serum and globulin levels comparable to those of adult sera

that had a good activating capacity. A more complete chemical study of the serum protein fractions may reveal other differences. For examination of sera of Rh blocking antibodies, only those infants' sera that showed a capacity to agglutinate sensitized cells equal to that of adult sera should be used.

In using plasma as a medium for Rh testing, Diamond and Denton[33] had trouble with rouleaux formation. This interfered with the macroscopic and microscopic reading of the test and with the determination of the end point in carrying out titrations. Human and bovine crystalline albumin gave reactions that were as sensitive as those with plasma, and were superior to those of plasma in that the clumping was firmer and more distinct, and there was no tendency toward rouleaux formation. Since bovine albumin was more available and cheaper, this was the medium of choice.

The critical concentration of albumin was a 20 per cent solution. Below this concentration reactions were weak or absent. Above 30 per cent viscosity made the solution unusable. A 30 per cent solution was the concentration of choice. Good agglutination occurred when the cell concentration was varied from 2 to 50 per cent. The 2 per cent was easiest to use and read. When the influence of salt content was investigated, hypotonic solutions of albumin pro-

duced swelling and hemolysis of the cells, but isotonic and hypertonic solutions gave good results. When the rate of antigen-antibody reaction was studied, agglutination was grossly visible in 1 minute and completed in 15-30 minutes at 37 C. At room temperature completion of the reaction occurred between 30 and 60 minutes. Sera containing only agglutinins, only blocking antibodies, and a combination of the two were compared, using both saline and albumin technics. If only agglutinins were present, the saline and albumin tests gave approximately the same titer. If only blocking antibodies were present, no reaction occurred in saline but strong agglutination took place in albumin. If a combination of the two were present, this was indicated by a difference in titer between the saline and albumin tests.

The effect of cerebrospinal fluid on the reaction between Rh antibodies and their antigens was studied by Jacobowicz and Bryce.[68] Human sera containing agglutinins with titers ranging from 1:64 to 1:2,000 were obtained from mothers of erythroblastotic infants. Blocking antibodies were present in 2 of the 6 sera. When 15 different specimens of cerebrospinal fluid were substituted for saline, 3 sera did not agglutinate Group O Rh positive cells, 1 was completely inhibited by 6 and partially inhibited by 2 of the 15 cerebrospinal fluids, and 2 anti-Rh sera were par-

tially inhibited. Since the 6 sera were not affected by the cerebrospinal fluids to the same degree, this would indicate that the inhibition of agglutination was partly due to a variable factor in the different specimens of serum. When the cerebrospinal fluid was replaced by saline, strong agglutination of the cells occurred. Thus, the cerebrospinal fluid had no effect on the Rh positive cells, but interfered with some component of the serum. When cerebrospinal fluid was used to dilute the anti-Rh serum, and the cell suspensions were made up in human serum or 25 per cent human albumin, the effect of the cerebrospinal fluid was partially counteracted by the serum and completely eliminated by the albumin. Later additions of human serum to tests carried out with cerebrospinal fluid caused the reappearance of agglutination, indicating that the inhibition is reversible, at least to a certain degree. Cerebrospinal fluids from rabbits and from a rhesus monkey gave similar inhibition. No inhibition was observed with artificially prepared fluids containing the main known chemical components of cerebrospinal fluid or with the chemically similar allantoic fluid from chick embryos. The factor in cerebrospinal fluid causing inhibition is heat stable. It was not destroyed by 56 C or 62 C for 1/2 hour or boiling for 5 minutes.

Gelatin.- Diamond and Denton[33] found that their solu-

tions of gelatin clumped both Rh positive and Rh negative cells. Levine made the proviso that certain varieties of gelatin could be used for direct testing for blocking antibodies. Fisk and McGee[43] found a special brand of gelatin satisfactory both for the determination of the presence of agglutinins and/or blocking antibodies and for titrations of the antibodies. This method was used to test 96 sera from Rh negative women. Thirty-two of the specimens were positive. These were found to contain blocking antibodies in varying titers and several contained agglutinins. The 64 negative sera were also negative when saline and plasma were used as the test media. Twenty antisera were titrated simultaneously using gelatin and plasma. The 2 media gave parallel results.

In 1945 Coombs, Mourant and Race[22] published a preliminary note describing an indirect and a direct test for detection of incomplete or blocking Rh antibodies. The same year a more detailed study appeared.[23] The essential mechanism of their test is based on the reaction of an anti-antibody against antibody specifically attached to red cells, as seen below. The direct test is useful in determining whether red cells have been sensitized <u>in vivo</u> by the incomplete antibody. The test was referred to in the original communication of Coombs, Mourant and Race[22] in 1945, and was described more fully in 1946.[25] In the

indirect test the materials were set up as for the detection of agglutinins. If there was no evidence of agglutination, the cells were washed 3 times with saline to remove all human serum and resuspended in 2 drops of saline. Two drops of an appropriate dilution of rabbit's anti-human-globulin serum previously absorbed with a mixture of human Group A, B and O cells were added and the test reincubated at 37 C for 1/2 hour. The tests were then read. If the cells were sensitized with incomplete Rh antibodies in the first part of the test, the rabbit's anti-human-globulin serum reacted with the human globulin on the red cells and cause clumping. Rabbits' sera reliable for the test after absorption with Group A, B, and O cells were; rabbit's anti-human pseudoglobulin, rabbit's anti-human-globulin, and rabbit's anti-human-whole-serum sera. A more detailed study appeared in 1947[26] in which both the antigen injected and the injection schedules of the rabbits were varied. The criterion used for an agglutinating dose was the smallest amount of antisera giving a 2 + reaction. For routine testing, 8 agglutinating doses were used. Group O serum was used to eliminate the production of undesirable anti-A and anti-B antibodies in response to dissolved A and B antigens in human seraof the other groups. Anti-alum-precipitated-human-serum sera were satisfactory. Fewer injections were necessary to produce a high titer. Normal

rabbits' sera had no effect on the sensitized cells. The reaction was not due to a nonspecific colloidal effect of immune sera in general, for high-titered rabbit's anti-typhoid and anti-pneumococcus Type 2 sera had no effect on sensitized cells.

Rabbits' antisera were prepared against albumin, alpha and beta globulin, and gamma globulin from human serum. After inactivation at 56 C for 1/2 hour and absorption with Group A, B and O cells, the antisera were tested to see whether they were specific for their respective antigen fractions by the precipitin flocculation test. Before and after neutralization with the different protein fractions of human serum, the antisera were tested for their ability to agglutinate red cells sensitized with incomplete Rh antibody. Antisera against different protein fractions of human serum were not specific for their respective fractions when examined by the precipitin flocculation test. When albumin was used to neutralize the different sera, there was no reduction in agglutinating titer of the sensitized cells. Neutralization with gamma globulin completely removed the ability of the sera to agglutinate sensitized cells. When alpha and beta globulin were used to neutralize the sera, only a slight reduction of titer occurred. The authors suggested 2 possibilities for this reduction. One was that there might have been traces of gamma globulin

in the alpha and beta globulin used for neutralization, the second, that a small part of the Rh antibody might be contained in the alpha and beta globulin fractions of human serum. From this serological evidence it appeared that the antibody in rabbit's anti-human-globulin serum which agglutinated red cells sensitized by incomplete Rh antibodies was an anti-gamma-globulin antibody, and that the incomplete antibody was present in the gamma fraction of human serum. It could not be stated that a small amount of incomplete antibody was not contained in the alpha and beta globulin fractions. Further evidence for the presence of the incomplete Rh antibody in the gamma fraction of human serum was obtained from chemical studies. Human serum containing incomplete antibodies was treated with ammonium sulfate. The fraction precipitated at 33 per cent saturation, which contains most of the gamma globulin, contained most of the incomplete antibody. The fraction precipitated between 33 and 50 per cent which contains most of the alpha and beta, as well as some gamma globulin, also sensitized red cells, but not as strongly as the first fraction. The albumin fraction of the human serum did not sensitize the cells at all. The above data would indicate that the incomplete antibody was contained mainly, if not entirely, in the gamma globulin fraction of human serum. Simmons[126] published a report on the use of rabbits' precipitating antisera to species other than human,

for agglutinating human red cells sensitized by incomplete Rh antibodies. The following antisera prepared in rabbits gave positive results with sensitized red cells and negative results with normal cells: anti-human, anti-porcine, anti-feline, anti-equine, anti-caprine, anti-canine, and anti-bovine. Anti-galline gave negative results.

The elution of antibodies from cells to which they are attached and demonstration of the antibodies in the supernate is a method which can be used to indicate the presence of agglutinins and/or blocking antibodies absorbed onto the surface of red cells. The elution technic is that of Landsteiner and Miller,[75] and adapted to the study of Rh antibodies by Haberman and Hill.[58] A somewhat different modification was suggested by Carter and Loughrey.[15] As indicated in both papers, agglutinins could only be obtained from cells of Rh positive infants born to Rh negative mothers in whose serum Rh agglutinins were demonstrable. Wheeler and Scholl[137] carried out a more detailed study on the optimal conditions for the release of Rh agglutinins from the red cells. When varying the pH of the saline used for suspending the red cells from 6.3 to 7.8, they found that the best results were obtained when the pH was at 7.0. The actual range of temperature, from 45 to 55 C, had little influence on the amount of antibody released. 45-50 C was preferable because

the amount of hemolysis was much less at 50 C or below. The duration of heat treatment was important in relation to the amount of antibody released. An increasing amount of agglutinin was released by 10-30 minute exposures to heat, accompanied by increase in hemolysis. For routine testing a 30-40 minute heating interval was used.

No incomplete or blocking antibodies could be demonstrated by the above technic. However, Wheeler and Scholl[137] found that if a 20 per cent solution of bovine albumin was substituted for saline during heat treatment and for suspending the test cells, blocking antibodies could be demonstrated in the eluate.

Stimulated by the lack of Rh typing antisera, Chown[20] devised the capillary method of Rh testing. Berlin[2] published a modification of this method. Since no saline or other extraneous crystalline solution was used, this method, if known cells and unknown serum were used, would indicate the presence of both agglutinins and blocking antibodies. Krieger and Weiden[74] found this method unreliable. When compared simultaneously with the Simmons slide test, there were 58 discrepancies out of 150 bloods tested. The method was also investigated in Boyd's laboratory and found to be very difficult to read.

The specific hemolytic action of Rh and Hr agglutinins and blocking antibodies (their "cryptagglutinoids") on corresponding red cells in the presence of complement was demonstrated by Hill and Haberman[65] and later by Hill, Haberman and Jones.[66] Little hemolysis occurred without the addition of complement. The amount of hemolysis was increased by gently shaking the mixture of anti-Rh serum, Rh positive blood and complement. In comparing the effect of different titers of both agglutinins and blocking ("cryptagglutinoids") antibodies, they observed that as the titers of the two kinds of antibodies were increased, "the hemolytic effect became more marked with relatively good correlation of titer and hemolysis."

Nature of Rh Antibodies.- Both agglutinins and blocking antibodies are proteins, Diamond and Abelson[34] found that they are precipitated by heat or alcohol. That they are both globulins was shown by chemical fractionation studies, ammonium fractionation studies, and by Witebsky's method for the concentration of Rh agglutinins. All studies agree that the agglutinins are present in the gamma fraction, but disagree as to the location of the blocking antibodies. The chemical fractionation studies,[36] in which Cohn's methods[21a] have been applied, indicated that the agglutinins were present in the gamma globulin fraction. Most of the blocking antibodies were found in the beta fraction, with

small amounts present in the alpha and gamma fractions. According to studies using different concentrations of ammonium sulfate,[26] the blocking antibodies are in the gamma fraction. This fraction contained most of the blocking antibodies. The fraction containing mostly alpha and beta globulins and some gamma globulin, yielded some blocking antibody, but less than the first precipitate. Dialysis of a human serum[162] containing both agglutinins and blocking antibodies separated the serum into 2 parts. The precipitate, which consisted mainly of globulins, contained the Rh agglutinins. The supernate, which was largely made up of albumin and some globulin, contained most of the blocking antibodies.

Coombs and Mourant[26] studied the chemical composition of the blocking antibodies by serological methods. These consisted of identifying the serum fraction needed to agglutinate red cells sensitized by blocking antibodies. They used the inhibition technic. Different fractions of human plasma were added to the rabbit-anti-human-globulin serum prior to addition to the sensitized cells. Neutralization by albumen did not reduce the agglutinating titer of the rabbits' antisera. The titer was completely removed by gamma globulin, but only slightly reduced by alpha and beta globulins. This was probably due to the presence of a small amount of gamma globulin precipitated along with the alpha and beta globulins.

A serum containing agglutinins and "developing antibodies" to a titer of 1:4,000 and a blocking titer of 1:2 (Wiener's original inhibition method) was studied electrophoretically.[66] The albumin and globulin fractions were tested for the presence of antibodies. These were found in the gamma globulin. It is to be regretted, in view of Diamond's localization of blocking antibodies in the beta globulin, that Hill, Haberman and Jones did not also study a serum high in blocking antibody content. It also would have been of interest to study sera containing antibodies demonstrable only by the Coombs test. Coombs and Race[24] found that Rh positive cells exposed to agglutinating and to blocking antibodies showed the same migration in an electric field. From this they concluded that the surface charge could not be the only factor causing clumping by agglutination. They also[24] found that blocking antibodies could not go through a collodion filter permeable to proteins whose molecular weight was 30,000A°. Ultracentrifugal studies seem to indicate that agglutinins are smaller than blocking antibodies.[36]

To summarize, agglutinins are gamma globulins. This is indicated chemically by chemical fractionation studies, fractionation studies making use of different percentages of ammonium sulfate, and by dialysis of serum. An electro-

phoretic study localized agglutinins in the gamma fraction. Serologically antibodies demonstrated by the Coombs technic were found in the gamma globulin fraction. Blocking antibodies have been localized by Diamond in the beta globulin fraction.

Agglutinins and blocking antibodies react differently to various physical agents. When exposed to heat blocking antibodies are more stable than agglutinins. Diamond and Abelson[34] found that prolonged exposure to 56 C would inactivate anti-Rh agglutinins but have little effect on blocking antibodies. Coombs and Race[24] found that blocking antibodies would withstand 65-70 C for 5-10 minutes. Wiener and Handman[157] made use of this property in their method for detecting blocking antibodies in sera containing a combination of the two kinds of Rh antibodies. Exposure of an alliquot of the serum under examination to 60 C for 1/2 hour completely destroyed the agglutinins, but resulted in only moderate deterioration of the blocking antibodies. Boyd[8] exposed anti-Rh agglutinins to pressures of 3,000-4,000 atmospheres. Agglutinating ability was destroyed, but such pressures did not convert agglutinins into blocking antibodies. Higher pressures of 4,500 to 5,000 atmospheres were required to destroy blocking antibodies. Photo-oxidation of anti-Rh agglutinins in the presence of eosin - Y as

sensitizer destroyed their agglutinating ability in 15 1/2 hours.[9] There are conflicting reports on the effect of merthiolate solutions on agglutinins. Waller,[134] and Diamond and Abelson[34] found that anti-Rh agglutinins are inactivated and blocking antibodies are not affected. However, Landsteiner and Wiener[79] added merthiolate to their guinea pigs' anti-rhesus agglutinins as a preservative, and Simmons and Graydon[124] found that Rh agglutinins to which merthiolate had been added were active many years later. When saline is present agglutinins will cause clumping of the red cells. Blocking antibodies will coat, but not clump the red cells in saline when the proportion of saline diluent to the total content of the tube reaches approximately 1/3.[33] Both agglutinins and blocking antibodies will cause agglutination of Rh positive red cells in various colloidal solutions.

Agglutinins and blocking antibodies also differ in their immunological characteristics. Agglutinins are found in humans immunized by transfusion of blood incompatible for the Rh factor or by immunization of a mother whose fetus has incompatible red cells. They are also found in the serum of animals who have responded to injection of Rh positive red cells. So far, blocking antibodies have been found only in humans. Diamond and Denton[33] stated that one of

their guinea pig-anti-rhesus sera showed a significant amount of blocking serum. This was based on the fact that the clumping of cells was much stronger in albumin than in saline. However, there was only a one-tube difference in titer which is within the error inherent in the method. Most workers state that agglutinins are "early" or "immature" antibodies, and that blocking antibodies are "late" or "mature" antibodies. This is based on the early appearance of agglutinins and the later appearance of blocking antibodies in actively immunized humans.[33] However, when Van Loghem[97] actively immunized Rh negative male volunteers, one exception was found in whom only blocking antibodies were formed without preceeding formation of agglutinins. It has been implied that blocking antibodies can be found in the serum of an individual longer than agglutinins. No careful studies of various individuals over a long period of time have been carried out. Since the kinds and amounts of antibodies are so variable among individuals, a large enough group would have to be followed to obtain statistically significant results.

The 2 types of antibodies also evidence different serological characteristics. If both Rh agglutinins and Rh blocking antibodies of the same specificity are present in a serum, the blocking antibodies react more rapidly than agglutinins

with their antigen on the red cell.[34] This is observed if there is not a masking of the blocking antibodies by a much greater concentration of agglutinins, when the test is done in saline.[157] Another indication is the number of absorptions needed to remove specific antibodies from a serum. Levine and Waller[95] found that blocking antibodies could be removed by a single absorption with specific red cells. Several treatments were necessary for the complete removal of agglutinins. Both agglutinins and blocking antibodies are reversibly attached to the red cells since the application of heat will cause the release of antibody into the supernate.[15,58,137] According to Witebsky[163] the strength of agglutination of red cells by blocking antibodies rarely reaches the strength of agglutination of red cells by strong agglutinins. Wiener[157] has stated that the specificity of the red cells involved differs. Cell aggregates caused by agglutinins are specific, that is, they contain only 1 type of cell, whereas clumps formed by blocking antibodies contain more than 1 type of cell. He also reported[157] that Rh agglutinins do not fix complement and lyse cells, but that blocking antibodies do. However, Hill, Haberman and Jones[66] found that agglutinins and blocking antibodies cause specific hemolysis of red cells in the presence of complement under the conditions of their experiment.

Whether agglutinins and blocking antibodies differ in their clinical significance has not been determined. Wiener stated[147] that the placenta is permeable to "univalent" blocking antibodies, but holds back "bivalent" agglutinins. Blocking antibodies could be found in the blood of a high percentage of infants whose mother's serum contained blocking antibodies, but agglutinins have been demonstrated in infants' bloods. It is quite possible that Wiener's impression is caused by the fact that blocking antibodies are found more frequently in maternal sera. He also observed[149] that blocking antibodies are the major cause of erythroblastosis fetalis due to Rh incompatibility between mother and infant, agglutinins being a very minor cause of this condition. However, not all workers in the field share this viewpoint.

Both Hill and Haberman, and Wiener believe that they have evidence for a third order antibody. Hill and Haberman[66] observed differences in the demonstration of antibodies and in their strength when testing sera by the agglutination, inhibition, and Coombs technics, and they thought that the difference between the three tests indicated that the basic character of the Rh antibodies must be different.

Hill, Haberman and Jones have presented date based on: (1) studies on mothers with erythroblastotic infants; (2) anti-

body patterns found during deliberate hyperimmunization; (3) investigation of changes in antibody characteristics during deterioration *in vitro* over a period of time; (4) the demonstration of the hemolytic action of the Rh antibodies, especially the third order or "cryptagglutinoid" type to establish their antibody nature; and (5) electrophoretic studies to determine whether agglutinins and "cryptagglutinoids" were in different protein fractions. Evidence for a third order antibody was presented on all points, but no proof has been demonstrated. A statement made by these authors seems to the writer so applicable to the field of Rh antibodies in particular that it is quoted in full. "It would seem apparent that the forces involved in the antigen-antibody reactions are so closely linked to physical chemistry that attempts to represent pictorially these activities on the basis of the knowledge at hand is pure speculation."

Wiener's[155] evidence is based on the ratios of titers when determined by the inhibition or indirect test for blocking antibodies, by the direct test for blocking antibodies using plasma as the medium, and by the direct test for blocking antibodies using albumin-plasma as the medium. The existence of a third order antibody awaits proof.

Three methods of concentrating Rh antibodies have been used. The method carried out in Cohn's laboratory is too involved for general use. Two other methods, one suggested by Witebsky, Mohn, Howles and Ward[162] and the other by Coombs, Mourant and Race[24] can be applied in any laboratory.

Witebsky's method is useful for concentrating agglutinins and for partly removing blocking antibodies that may be present in the same serum. The procedure is to dialyze serum containing Rh agglutinins. The precipitate is composed mainly of globulins. It is sedimented and redissolved in 1/10 the original volume of saline. This method can be used to make ordinarily weak sera usable for diagnostic typing.

Coombs, Mourant and Race's method is based on the fact that saturation of the serum with a 25-33 per cent ammonium sulfate will precipitate most of the gamma globulin.

The specificity of the Rh antibodies is indicated below. In animals only agglutinins corresponding to anti-D have been demonstrated.

Humans differentiate among the various Rh antigens much more readily than animals. This is indicated by the

wide varieties of Rh antisera produced by the introduction of cells incompatible for the Rh factor into the blood stream of man. The following are Rh antisera that have been demonstrated to date:

anti-C, anti-C^w, anti-C^u

anti-c, anti-c^v

anti-D, anti-D^u

anti-d

anti-E

anti-e

The reactions of humans to Rh antigens are dealt with more fully in the section under Rh antigens.

PRESENT INVESTIGATION

Rationale.- The general conclusion which emerges from a survey of the present literature is that the Rh factors, at least when administered in the usual way, are poor antigens in experimental animals. The most potent animal antisera obtained cannot approach the strength and avidity of good human anti-D sera. In man the best anti-Rh sera are produced by mothers of erythroblastotic infants. If one assumes that the fetal antigen enters the maternal circulation at least during the latter part of the pregnancy, an ideal situation for immunization is present. The reactive organism is continuously stimulated by small amounts of antigen over a long period of time.

Increasing use has been made of adjuvants to enhance antigenic stimulation. When the antigen is suspended in the water phase of a water-in-oil emulsion it seems to be released more slowly and over a long period of time. Mudd and Smolens injected mice subcutaneously with Shigella antigen in a water-in-oil emulsion. Material from the site of injection was introduced into fresh mice after various time intervals. They found that the amount of the deposited vaccine diminished within 2 weeks, but that traces of the antigen were still demonstrable 22 weeks after injection. Freund, who has investigated the role of both paraffin oil

and tubercle bacilli in water-in-oil emulsions, observed that paraffin oil increased and prolonged antibody formation, but had little effect on sensitization, and that Mycobacteria increased antibody formation and altered sensitization.

Adjuvants have been successful in stimulating antibody production in a number of situations. Their use has increased the titer and prolonged the activity of antibodies against the typhoid bacillus and against the diphtheria toxoid. However, the addition of tubercle bacilli exerted little effect over and above that contributed by the paraffin oil and emulsifier. Water-in-oil emulsions containing tubercle bacilli have also been effective in immunizing against influenza and Japanese B encephalitis viruses, against epidemic and murine types of typhus vaccines, and against malaria. Antibody formation to poor antigens such as alcoholic extract of brain, histaminazo protein, and ragweed pollen was enhanced by suspending the antigen in the water-in-oil emulsion containing tubercle bacilli.

Since the Rh antigens are poorly antigenic, and since antibody formation by other weak antigens has been enhanced by incorporating them into the water phase of a water-in-oil

emulsion containing tubercle bacilli, the following attempts have been made to produce usable Rh antibodies in animals, employing adjuvants with the poorly antigenic Rh antigens.

EXPERIMENT I Macacus rhesus erythrocytes as the antigen.

Selection of Animals.- Smooth-haired guinea pigs (550-950 g) were divided according to the weight and color as evenly as possible into two groups of 5 each. Group 1 received whole rhesus red cells incorporated into the water-in-oil emulsion. Group 2 received rhesus stroma in the emulsion. Also rabbits (8-10 lbs.) in 2 groups of 5 each received similar injections.

Preparation of Antigens.- Rhesus erythrocytes suspended in sodium citrate were kept in the refrigerator for 5 days before use. Preliminary tests on the technic of preparation of antigens were made first with the more readily available horse erythrocytes. For the preparation of stroma partial use was made of Heidelberger's[61] method but freezing and thawing were eliminated, for nothing was known of the action of these procedures on the Rh antigen. 100 ml of defibrinated horse blood was centrifuged in the refrigerated centrifuge at 40 F at a speed of 4500 r.p.m. for 10 minutes. The plasma was removed and the cells washed

3 times with 0.9 per cent NaCl. 41 ml of packed horse erythrocytes were poured into 10 volumes of chilled distilled water saturated with CO_2 while chilled. The material was centrifuged as above and the supernate removed. The volume of the pink viscous sediment was still 41 ml. The material was then washed 5 times with 0.2 per cent NaCl. At the end of the procedure it was puzzling to find that the volume was still the same. As a check the same procedure was carried out with fresh human erythrocytes with similar results.

The rhesus erythrocytic antigens were prepared as above, using sterile precautions. After 3 washings with 0.9 per cent NaCl, 20 ml of whole packed red cells were set aside for Group 1 of the guinea pigs and Group 1 of the rabbits. The remaining 52 ml of packed rhesus erythrocytes were poured into 10 volumes of chilled sterile distilled water. The sedimented stroma were used as antigen for Group 2 of the guinea pigs and rabbits. No Sharples centrifuge was available at the time. Again, using rhesus erythrocytes, there was no change in volume following treatment of the red cells with distilled water.

Preparation of Emulsion.- See p.75 under Experiment II.

Injection Schedule.- Groups 1 and 2 of guinea pigs

TABLE III

Experiment I

Material Injected per Animal

Group	Total amount material injected ml	Whole packed red cells ml	Stroma ml	Falba ml	Bayol F ml	t.b. mgm
1	3	1.2	—	0.6	1.2	0.2
2	3	—	1.2	0.6	1.2	0.2

and rabbits received the same injection schedule. Two series of inoculations were given. On 3 successive days of the first week of the experiment each animal received 0.5 ml of emulsion in the nuchal regions, alternating between right and left. Each 0.5 ml was divided into two 0.25 ml injections given simultaneously.

Bleeding Schedule.- See p. 75 under Experiment II.

EXPERIMENT II Human Group O, MN, Rh -/- erythrocytes as the antigen.

Selection of Animals.- Smooth-haired guinea pigs (500-1,000 g) were distributed according to weight and color as uniformly as possible in 6 groups of 10 each. The antigen given to Groups 1, 2, and 3 was prepared from whole packed human Group O, type MN, Rh -/- erythrocytes. Stroma prepared from the same pooled cells were injected into Groups 4, 5, and 6. Groups 1 and 4 were injected intraabdominally with plain antigens, Groups 2 and 5 subcutaneously with the antigen in a water-in-oil emulsion, and Groups 3 and 6 received the emulsion intraabdominally.

Preparation of Antigens.- Four liters of human Group O, type MN, Rh -/- (all 3 Rh antigens, rh', Rh^{o}, rh" (C, D and E) were present) erythrocytes pooled from 18 separate individuals were used as the antigen. The whole packed red

TABLE IV

Experiment II

Groups of Guinea Pigs Used*

Material injected		Form	Route
Whole cells**	Stroma		
Group 1	Group 4	Plain	Intraabdominal
Group 2	Group 5	Emulsion	Subcutaneous
Group 3	Group 6	Emulsion	Intraabdominal

* 10 animals in each group

** see text

cells were prepared by washing the cells 3 times in 0.9 per cent NaCl in a refrigerated centrifuge at 4,000 r.p.m. for 10 minutes at 40 F. Stroma were prepared from the washed erythrocytes by suspending the latter in distilled water which was then run through a Sharples Centrifuge to collect the stroma.

Preparation of Emulsion.- Freund's method[49,131] of preparing the emulsions was used. Two parts of the antigen were mixed with 1 part of Falba in a mortar,[7] and the mixture then combined with 2 parts of Bayol F containing killed, dried *M. tuberculosis* Jamaica #22.

Injection Schedule.- Groups 1 and 4 received 1.5 ml of antigen per week intraabdominally for the first 5 weeks, 0.5 ml being given on 3 successive days of each week of injection. Groups 2 and 5 received 1 dose of 2 ml of emulsion subcutaneously, divided into 4 separate injections of 0.5 ml, 2 of which were injected into the left nuchal region and 2 simultaneously into the right. Groups 3 and 6 received 1 dose of 2 ml of emulsion divided into 2 injections of 1 ml each, which were introduced simultaneously into the right and left sides of the intraabdominal cavity. See Table II for the total amount of material injected.

Bleeding Schedule.- At each bleeding 5 ml of blood

TABLE V

Experiment II

Material Injected per Animal

Group	Total amount material injected ml	Whole packed erythrocytes ml	Stroma ml	Falba ml	Bayol F ml	t.b. mgm
1	7.5	7.5	—	—	—	—
2 and 3	2	0.8	—	0.4	0.8	0.2
4	7.5	—	7.5	—	—	—
5 and 6	2	—	0.8	0.4	0.8	0.2

were obtained under aseptic conditions by cardiac puncture. Samples were obtained at 0, 1, 2, 3, 4, 6, 8, 10, and 12 weeks from the beginning of the experiment. Since the injections of Groups 1 and 4 were continued through the fifth week, they were also bled at 5, 7, and 9 week intervals. The clotted blood remained overnight in the refrigerator and the serum was then separated aseptically. The sera were inactivated by heating to 56 C for 20 minutes, and then kept without preservative in the refrigerator.[134]

Testing of Sera.- In Experiment I the guinea pigs' and rabbits' anti-rhesus sera were tested for the presence of agglutinins. In Experiment II the guinea pigs' anti-human Rh sera were examined for agglutinins and blocking antibodies.

Agglutinins.- The basic method for testing for agglutinins was as follows: Each serum was tested against 4 different red cell suspensions, 2 Rh +, 1 CDe and 1 cDE and 2 Rh negative (cde), keeping the source of erythrocytes as constant as possible. A 2 per cent suspension was obtained by adding whole blood to 0.9 per cent NaCl, centrifuging, removing the supernatant, and resuspending the cells to 2 per cent volume. One drop of serum was added to each of four 75 x 9 mm blood grouping tubes. A drop of the appropriate cell suspension was added to the tube, the mix-

ture shaken and incubated in a 37 C water bath for 1/2 hour, The tubes were then centrifuged at 500 r.p.m. for 30 seconds and examined microscopically using a magnifying mirror. Those that showed questionable or no clumping were examined microscopically. Known positive and negative controls were used each day. The positive serum control was human anti-D serum and the negative control was normal guinea pig's serum which contained no agglutinins against human erythrocytes.

Three procedures were used in testing for agglutinins. First the undiluted sera were tested for agglutinins to the erythrocytes. One drop of the undiluted serum in this case was added to each of the 4 test tubes. Positive sera were diluted with 0.9 per cent NaCl in steps of 10 and tested against the same cell suspensions to determine the strength of agglutinin production, and to see whether Rh -/- and Rh negative cells could be differentiated by dilution of the serum. Undiluted sera that showed a minimum of 2 -/- clumping with Group O Rh -/- erythrocytes were then absorbed with packed human Group O, type MN, Rh negative red cells to test for the presence of specific Rh antibodies.

Blocking Antibodies.- After the erythrocytes to be used for the test Rh -/- and Rh negative suspensions had been washed in 0.9 per cent NaCl 3 times, a 2 per cent

suspension of the cells was made up in 30 per cent bovine albumin. Each serum was tested against the 4 separate cell suspensions, 2 of which were Rh + and 2 Rh negative. One drop of the serum to be tested was placed in each of four 75 x 9 mm blood grouping tubes. One drop of the appropriate cell suspension was added, the contents mixed, and incubated in a 37 C water bath for 1 hour. The tubes were then centrifuged at 500 r.p.m. for 1 minute and read macroscopically. Those that showed questionable or negative reactions were checked microscopically. The system was checked with known human anti-D blocking serum.

Specific Rh Antibodies.- Absorptions were carried out in the same way whether for Rh agglutinins or blocking antibodies. When absorbing for agglutinins, the sera were diluted 1:10 with 0.9 per cent NaCl (1:10 had been found to be as suitable as undiluted or 1:5). When absorbing for blocking antibodies, sera were used undiluted since only a limited amount of bovine albumin was available. In general, for each absorption 1 ml of serum was in contact for 1/2 hour with 0.1 ml of packed human Group O, type MN, Rh negative erythrocytes which had been prepared as described for the preparation of antigen. The suspension was then centrifuged, the supernatant removed, and tested for either Rh agglutinins or blocking antibodies,

as described above. If after 3 absorptions no Rh antibodies had been obtained, absorption of that particular antiserum was discontinued, for it was found that a continuation of the procedure yielded no positive results.

Criteria for Evaluating Antisera.- In this study, an adequate antiserum was defined as one that shows at least a two step difference (Table IV) in strength of reaction between Rh +/- and Rh negative cells (e.g., 2 +/- with the 2 Rh +/- cell suspensions and — with the 2 Rh negative cell suspensions). Those sera that showed a 1 or 1 1/2 step difference were called weak (e.g., 2 or 2 1/2 +/- with Rh +/-, 1 with Rh negative cells).

Results.-EXPERIMENT I.- In Experiment I the agglutinins produced by the injection of rhesus erythrocytes incorporated in a water-in-oil emulsion into guinea pigs and rabbits had disappeared by the time the sterile sera had been in the refrigerator for a month and a half. Similar observations were made with sera from guinea pigs and rabbits injected with rhesus stroma incorporated into an emulsion. Due to the disappearance of these agglutinins, no information could be obtained on the strength or specificity of the agglutinins or on the presence of blocking antibodies. Such an observation would indicate that the rhesus erythrocytes incorporated into a water-in-oil emulsion are unsatisfactory as antigenic material for the production of Rh agglutinins.

Results.- EXPERIMENT II.- As shown in Table III, of the 3 groups of guinea pigs injected with packed human Group O, type MN, Rh -/- erythrocytes (Group 1, 2, and 3), adequate agglutinins were produced only in Group 1, in which the plain antigen had been injected intraabdominally. Six of the 10 guinea pigs produced adequate agglutinins, and 9 produced weak Rh agglutinins. One animal in Group 3, which received the antigen suspended in emulsion intraabdominally, produced weak antibodies. Group 2, injected subcutaneously with the antigen-emulsion, produced no Rh agglutinins. Of the 3 groups injected with stroma prepared from the above Group O, type MN, Rh -/- red cells (Groups 4, 5, and 6), 1 animal in Group 4, which received the plain stroma intraabdominally, produced adequate agglutinins and 1 showed weak agglutinins. One animal in Group 6, which received the antigen-emulsion intraabdominally, showed weak antibodies. Group 5, which received the antigen-emulsion subcutaneously, behaved similarly to Group 6.

The time of appearance of specific Rh agglutinins (see Table IV) differed markedly from the peak of general agglutinin production. Six of the 7 adequate Rh antisera (6 from Group 1, 1 from Group 4) were obtained from guinea pigs 1 week, and one 2 weeks after the first injection of antigen. Antisera obtained 3, 4, 6, 10, and 12 weeks after

TABLE VI

Experiment II

Distribution of Adequate and Suggestive Rh Antisera

Group	Antigen	Route	Number of Guinea Pigs Producing Adequate Rh Antisera	Suggestive Rh Antisera	No Rh Antisera
1	packed erythrocytes	i.a.*	6	9	—
2	erythrocytes in emulsion	s.c.**	—	—	10
3	erythrocytes in emulsion	i.a.	—	1	9
4	stroma	i.a.	1	1	8
5	stroma in emulsion	s.c.	—	1	9
6	stroma in emulsion	i.a.	—	1	9

* i.a. signifies intraabdominal injections

** s.c. signifies subcutaneous injections

the injections were started showed no Rh agglutinins. (Injections of the antigen were continued through the fifth week). Thirteen guinea pigs produced weak agglutinins; 1 after the first and second weeks of injection, 1 after the first week and not subsequently, 10 two weeks after, and 1 the fourth week after injections were started.

When the sera of the 6 experimental groups had been tested for the presence of general anti-human Group O, type MN agglutinins, and for their strength, when present, by titration in steps of 10, all 6 groups showed essentially the same results. The titer was the same when tested against the 4 separate suspensions of erythrocytes, 2 Rh +/- (CDe and cDE) and 2 Rh negative (cde). Sera obtained 1 and 2 weeks after the first injection, which contained all 7 of the adequate and 13 of the 14 weak antisera, showed a titer to the erythrocytes as a whole of undiluted to 1:1,000, the majority at 1:100. The peak of general agglutinin production to the whole red cell or stroma occurred from the fourth to the sixth week, primarily at the former. The peak titer in no case went above 1:10,000. There was no relationship between titer and later demonstration of specific Rh agglutinins upon absorption. The strength of differentiation between Rh +/- and Rh negative erythrocytes by the Rh agglutinins is indicated in Table IV.

TABLE VII

Experiment II

Summary of Strength of Rh Agglutinin Reactions of Guinea Pig Antisera

Adequate Rh Agglutinins						
Group	Guinea pig Number	Week Number	Rh-/-	Rh -/-	Strength of Reactions Rh —	Rh —
1	1	1	2	2	—	—
	3	1	2	2	—M-/-	
	6	2	2	2	—M-/-	—M-/-
	7	1	2.5	2.5	—M—	
		2	2	2	—M-/-	
	8	1	2	2.5	—M-/-	
	10	1	2	2	—M—	
4	31	1	2	2	-/-M-/-	

TABLE VII Continued

Group	Guinea pig Number	Week Number	Weak Rh Agglutinins Rh -/-	Rh -/-	Strength of Reactions Rh —	Rh —
1	1	2	1	1	—	—
	2	1	2	2	1	1
		2	2	1	—M—	
	3	2	±M-/-	1	—M—	—M—
	4	2	2	1	—M—	
	5	2	2	1	—M—	—M-/-
	6	1	2	1	—M-±	—M—
	8	2	2.5	2.5	1	1
	9	2	2.5	2	1	1
	10	2	1	1	—M-/-	
3	29	4	1	1.5	—M—	
4	32	2	2	1.5	—M-/-	—M-/-
5	50	2	2	1	±M-/-	±M-/-
6	51	2	2	2	1	1

4 -/- all cells are agglutinated into one clump that could not be broken up upon gentle agitation

3 -/- button at bottom of tube broken up into a few large clumps

2 -/- button broken up into medium sized, well-defined clumps

1 -/- button broken up into small clumps that were just visible microscopically

± questionable reaction

— no reaction

M-/- positive microscopically

M— negative microscopically

Half step reactions indicate a strength between the two steps

Of the 2 technics used to determine the presence of Rh agglutinins, namely, absorption of non-Rh agglutinins with Group O, type MN, Rh negative erythrocytes, and dilution of the sera, only the former method was effective. In no instance did dilution of the serum in steps of 10 produce either adequate or weak agglutinins. No prezone phenomenon was observed.

Blocking Antibodies.- The sera were also tested for the presence of Rh blocking antibodies, that is, antibodies that will differentiate between Rh -/- and Rh negative cells when the erythrocytes are suspended in 30 per cent bovine albumin, but not when suspended in saline. No Rh blocking antibodies were obtained after absorption of the antisera with Group O, type MN, Rh negative red cells.

The unabsorbed sera from the first and second week bleedings were titrated in steps of 10 against red cells suspended in 30 per cent bovine albumin. There was no difference in titer between the sera when tested against cells suspended in 0.9 per cent NaCl and the albumin. Since agglutinin production was so poor, and no blocking antibodies were found, the more delicate Coombs[23] antibodies were not investigated.

DISCUSSION

As indicated by the above experiment, the total expression of antibody production to the Group O, MN, Rh positive erythrocytes as a whole was the same whether whole cells or stroma were employed as the antigen, and whether the antigen was suspended in saline or in a water-in-oil emulsion. Incorporation of the Rh positive red cells into the emulsion containing tubercle bacilli had an inhibiting effect on Rh antibody production in the guinea pig. Only whole red cells suspended in saline stimulated the production of Rh agglutinins. Contrary to the observations reported by Gallagher and Pillischer, no Rh agglutinins were obtained in guinea pigs inoculated with the stroma of Rh positive red cells prepared by Sharples centrifugation. At present no explanation can be offered for this discrepancy.

Numerous interpretations may be offered to explain the lack of response in the animals who received the Rh positive red cells in emulsion. As indicated in Table II the guinea pigs in the control group received a total of 7.5 ml of packed Rh positive erythrocytes. The groups injected with the Rh positive cells in emulsion received a total of 0.8 ml. At the time of peak Rh agglutinin pro-

duction the second week after injections were started, the animals in the control group had received 3 ml and the emulsion groups 0.8 ml of the packed erythrocytes. Even though at that time there was a 4-fold difference in the amount of antigenic stimulus, it is hard to accept the difference in Rh agglutinin production observed on a quantitative basis. Since all groups showed similar agglutinin production to the human red cells as a whole, some Rh agglutinins should have been found in the sera of guinea pigs injected with the antigen in emulsion.

A second possibility may be that the weak Rh antigens are masked more effectively by the stronger antigens when the red cells are suspended in the emulsion. This too seems unlikely, for if the antigen could get through to the antibody-producing cells, multiple antibody formation would be likely to occur. Since both saline and emulsion groups showed a similar titer of antibody to the total human erythrocyte, antigen must have penetrated to the cells that form antibodies.

A third possibility is that the inhibiting effect of the emulsion may be due to the chemical nature of the Rh antigen. Incorporation of the antigen into an emulsion may either destroy the Rh antigen or block it by either physical or chemical combination. Since, as pointed out

above, the antibody titer to the human Rh positive red cells as a whole is the same in both saline and emulsion groups, the cells or their disintegration products must have left the site of inoculation and come into contact with the antibody-producing cells. In view of this fact, the third possibility in our opinion seems to be the most plausible explanation.

The method of choice for any further experiments involving the use of guinea pigs for the production of Rh or Hr agglutinins in response to the injection of red cells would involve the inoculation of saline suspensions of the antigen. So far, the intraperitoneal route has been used exclusively for such injections. It would be of interest to compare this route with intravenous inoculations into the guinea pig.

The source of antigen for the production of Rh agglutinins in animals has been either rhesus or human Rh positive red cells. The erythrocytes of each species are antigens of great complexity. Only a small portion of the total antigenic stimulus is produced by the desired Rh antigen. For future work, a more concentrated or a pure antigen would be desirable. Calvin, Evans, Behrendt and Calvin[12] and Carter[17] have reported that a lipid fraction extracted from Rh positive human erythrocytes contains a

substance which inhibits human Rh antibodies. Carter found that the fraction alone was nonantigenic in guinea pigs. When combined with a "schlepper" (a potent antigen), 5 of 24 guinea pigs produced agglutinins to Rh positive human erythrocytes. Whether they were Rh agglutinins could not be determined from the report. Further experiments would have to be undertaken to determine the antigenicity of this lipid using varying immunization procedures. Witebsky and Mohn[161] isolated a substance from the amniotic fluid of some infants. This substance inhibited Rh antibodies, and when concentrated 10 times by lyophilizing the material and resuspending it in 1/10 the volume of saline, is even more effective. No studies have been published on the possible antigenicity of the material.

Since animal anti-rhesus sera cannot differentiate Rh positive from Rh negative infants, and since there is evidence to indicate that the human D and rhesus antigens are not identical, future antisera produced in animals should be stimulated by human antigens.

No information has been published on any attempts to produce Rh-Hr antisera other than anti-D agglutinins in guinea pigs. Since sera for subtyping in particular

are rare and the supply variable, it would seem that experiments investigating the possibility of producing these sera in animals should be undertaken. It would be very important to completely subtype all cells employed for both inoculation of the animals and absorption of the sera, to eliminate all but the desired agglutinins.

SUMMARY

1. Macacus rhesus erythrocytes gave rise to agglutinins for human erythrocytes in 5 guinea pigs and 5 rabbits injected subcutaneously with rhesus red cells in a water-in-oil emulsion. Similar results were obtained in the 5 guinea pigs and 5 rabbits that received rhesus stroma suspended in the emulsion.

2. Upon standing in the refrigerator for a month and a half after the initial testing, the anti-rhesus erythrocyte agglutinins had disappeared in the guinea pigs' and rabbits' sera.

3. Human Group O, type MN, Rh -/- erythrocytes gave rise to adequate Rh agglutinins in 6 of 10 guinea pigs injected intraabdominally with a plain cell suspension. Weak agglutinins were present in 9 of the 10 animals of this group.

4. When Rh -/- erythrocytes were incorporated into a water-in-oil emulsion with Falba, Bayol F and M. tuberculosis Jamaica #22, not only was there no enhancement of Rh antibody formation, but such agglutinin formation seemed inhibited. Thus, the adjuvants used did not enhance anti-Rh production but actually seemed to depress it.

5. When stroma were prepared by Sharples centrifugation of the above erythrocytes, the material was poorly antigenic when tested by injection of the plain stroma. Stroma incorporated into the above emulsion also produced no Rh agglutinins when injected into guinea pigs.

6. The peak of Rh agglutinin production was reached 1 to 2 weeks after weekly injections of the antigen were started. The peak of agglutinin production to the whole Group O, type MN, Rh -/- red cell and stroma was reached the fourth to sixth week after injection had been started.

7. Despite continued injection of the antigens, guinea pigs producing Rh agglutinins no longer possessed these antibodies after 2 weeks.

8. No Rh blocking antibodies were demonstrated.

9. Guinea pigs do not seem to produce anti-Rh agglutinins comparable to good human sera.

10. Using part of Heidelberger's method to prepare stroma from rhesus, human, or horse erythrocytes, the original cell volume and the final volume were the same.

11. Stroma prepared by Sharples centrifugation are concentrated to 10 times the initial volume.

Acknowledgments are due to Dr. Dwight Mulford, Miss Julie Sullivan, and Miss Betty Griffith of the Massachusetts Department of Public Health, Division of Biologic Laboratories for the human erythrocytes, to Dr. E.A. Kabat for the rhesus erythrocytes, to Dr. Jules Freund for the tubercle bacilli, to Dr. A.S. Wiener for the human Rh agglutinating serum, to Dr. L.K. Diamond for the Rh blocking aerum, to Pfaltz and Bauer, Inc., for the Falba, and to Stanco Distributors, Inc., for the Bayol F.

APPENDIX - TECHNICAL METHODS

I. Methods used with animal anti-Rh sera

Landsteiner and Wiener[78] 1941 - See P. 28

Immunization Procedure.- Washed red cells from 1 and later 2 ml of whole blood of monkeys were injected into the guinea pigs intraabdominally. Five days later the animals were given a second injection and 1 week later they were bled.

Testing for Agglutinins.- To test for the presence of agglutinins 2 drops of serum and 1 drop of a 2 per cent suspension were added to a small test tube, shaken, and allowed to stand at room temperature for 30 to 60 minutes, then read by direct examination of the sediment. After an hour at room temperature, they were reread and the negative tests checked microscopically.

Landsteiner and Wiener[79] 1942 - See P. 29

Modified Immunization Method.- Red blood cells from 2 ml of rhesus blood were injected 5 times at 5 day intervals. The animals were bled 1 week after the last injection. Satisfactory sera were pooled. Rh antisera were produced by absorbing sera diluted 1:10 with saline with 1/10 volume of equal parts Group A and Group B red cells.

Davidson and Toharsky[28] - See P. 29

Immunization Procedure.- The rhesus red cells were washed 3 times. The guinea pigs were injected intraabdominally with the cells from 1 ml of blood with 2 injections at 5-day intervals. They also gave rabbits 7 daily intravenous injections of 0.1 ml of the suspension, followed by a 3-week rest and then a second series of injections. The guinea pigs and rabbits were bled 1 week after the last injection of antigen.

Testing for Agglutinins.- In testing for Rh agglutinins 1-2 drops of serum and 1 drop of a 1 per cent red cell suspension in saline were added to a small test tube, shaken, incubated in a 37 C water bath for 1 hour, centrifuged for 1 minute at 500 r.p.m., then read, and checked microscopically.

McIvor and Lucia[104] - See P. 33

Immunization Procedure.- In following the first schedule, 20 guinea pigs were injected intraabdominally with the red cells from 2 ml of rhesus blood. Five days later a second injection was given. Six weeks later 5 animals of the above group were given a second series of similar injections. The third schedule was tried on 16 guinea pigs. Three inoculations, 5 days apart, were given. At this time injections could not be continued for the animals were hypersensitive.

Nine guinea pigs died soon after the third injection. The fourth schedule consisted of 5 intraabdominal injections at 2-day intervals, starting with 1 ml and increasing 0.5 ml with each injection. Bleedings were taken in the first 3 groups 7 days after the last injection of antigen. In the last series blood was taken 2 days after the last injection. To produce specific antisera, the serum was diluted 1:10 with saline, and immediately before use 0.05 ml of washed Group A cells and 0.05 ml of washed Group B cells were added to 1 ml of the diluted serum. The mixture remained at room temperature for 1 hour. After absorption the serum was further diluted 1:3 with saline. In testing for agglutinins, 2 drops of serum and 1 drop of a 1.5 per cent cell suspension were placed in a small test tube, incubated at room temperature for 1 hour, then read.

Homburger[67] - See P. 34

Immunization Procedure.- The guinea pigs received washed cells from 1 ml of blood resuspended in saline, intraabdominally. They were injected twice with a 5-day interval between injections. The rabbits received 0.01 ml of citrated cells suspended in 1 ml of saline, intravenously. They were injected on 3 successive days, and 5 days later received a second series of injections.

Scherer[122] - See P. 34

Immunization Procedure and Testing for Agglutinins.- Nine animals were in the control group, 8 in the group injected with ethylene disulfonate, 6 in the group injected with pyrabenzamine, 8 in the group injected with sodium salicylate, and 7 in the group injected with A and B blood group substances, making a total of 38 rabbits. Three daily injections of 1 ml of 1:100 rhesus cells were given intravenously. This was repeated 4 days later. Bleedings were obtained before and at intervals during the experiment. Sera were inactivated at 52-56 C for 30 minutes. To eliminate agglutinins other than Rh, 1 ml of serum was absorbed with 5 ml of packed Group O, MN, Rh negative human red cells for 1 hour at room temperature. The sera were tested against Group O, M CDe/cDE red cells suspended in saline, and suspended in AB serum. Three drops of serum and 1 drop of a 2 per cent red cell suspension were placed in a small test tube, incubated at 42 C for 1 hour, centrifuged at 500 r.p.m. for 1-2 minutes, and read. The gradations of the reactions were: 4 +/- if the reaction was visible macroscopically, 3,2, 1, and +/- on the basis of the size of clumps visible microscopically.

Carter[14] - See P. 36

Immunization Procedure.- The cells were washed and 6

intraabdominal injections given at 2-day intervals. The first was 1 ml, the second 1.5 ml, and the rest 2 ml. The animals were bled 2 days after the last injection. The sera were pooled and absorbed with an equal volume of Group O, MN, Rh negative cells and inhibited with A and B substances.

Carter[16] - See P. 36

Immunization Procedure.- Six injections of 2 ml each were given to 24 guinea pigs every other day. The animals were bled 1 week after the last injection.

Gallagher and Pillischer[56] - See P. 37

Immunization Procedure and Preparation of Antisera.- The stroma were prepared by lysing the cells in distilled water and passing the suspension through the Sharples centrifuge. The stroma were washed until free of hemoglobin. The pale pink residue was repeatedly injected into guinea pigs intraabdominally. One week after the last injection the animals were bled and the sera inactivated at 56 C for 30 minutes. They were then absorbed with Rh negative red cells.

Carter[16] - See P. 37

Immunization Procedure.- The antigenicity of the lipid material was tested as follows. Twenty-four guinea pigs and 2 rabbits were inoculated with a 10 per cent suspension of

the lipid material. Six injections of 2 ml each were made on alternate days into the guinea pigs intraabdominally and into the rabbits intravenously. Bleedings were made 1 week after the last injection.

II. Technical Methods

Landsteiner and Wiener[78] - See P. 40

Original Method of Testing for Agglutinins.- Two drops of the test serum or serum dilution made up with saline were mixed with 1 drop of 2 per cent fresh red cells suspended in saline in a small test tube. The tests were allowed to remain at room temperature for 1 hour and then read by direct examination of the sediment. "Negatively reacting bloods then show a circular deposit with a smooth edge, while positive bloods show a wrinkled sediment with a serrated border or show a granular deposit." The tubes were then shaken and read again after 2 hours at room temperature. The sediment was again examined macroscopically and the suspension examined microscopically.

Diamond and Abelson[35] - See P. 43

Slide Test.- 0.2 ml of fresh oxalated Rh negative, Rh_1 and Rh_2 Group O whole blood or 40-50 per cent cell suspensions are placed on a glass slide. 0.1 ml of the serum to be tested is added to each blood. The mixture is tilted and read in 1-3 minutes. The reaction occurs a little more rapidly at 37 C, but can be carried out at room temperature.

Simmons[123] - See P. 44

Slide Test.- The Rous-Turner mixture used to suspend the red cells consists of 2 solutions. Solution 1 is made up of 16 ml of 5.4 per cent glucose in distilled water, solution 2, 6.6 ml of 3.8 per cent Na citrate in distilled water. Solutions 1 and 2 are autoclaved at 110 C for 15 minutes, then mixed together. A drop of a 5 per cent suspension of the patient's cells is mixed with the first, a drop of known Rh positive suspension to the second, and a drop of known Rh negative cells to the third of 3 drops of potent anti-D serum placed in separate rectangles drawn on a glass slide. The specimens are mixed and incubated for 30 minutes at 37 C, then gently rocked and readings made.

Witebsky, Rubin and Blum[163] - See P. 47

Method of Testing the Enhancing Property of Normal Sera on Anti-Rh Sera.- Decreasing amounts of the anti-Rh serum in a total volume of 0.1 ml were mixed with 0.1 ml of a 3 per cent suspension of Group O, Rh positive red cells. Saline, adult sera, cord sera, and premature sera were tested for their capacity to cause agglutination by the blocking antibodies. They were used both as diluent for the blocking sera and suspension medium for the red cells. The tests were incubated for 1 hour at 37 C, shaken slightly, centrifuged at moderate speed for 2 minutes, and read.

Diamond and Denton[33]

Albumin Blocking Test.- Two testing technics were recommended: a. Cell suspensions of Group O Rh_1 Rh_2 and Rh negative red cells washed 3 times were made up to 2 per cent in 20 per cent bovine albumin. One drop of undiluted serum was placed in each of 3 small tests tubes and 2 drops of the appropriate cell suspension was added. The mixture was shaken, incubated in a 37 C water bath for 5-10 — 30 minutes, centrifuged for 1 minute at 500 r.p.m., and read macroscopically. Negative reactions were confirmed microscopically. b. In running routine tests, if a test for agglutinins was negative, the material was centrifuged, the supernate removed and replaced by 2 drops of 20 per cent bovine albumin. The tube was shaken, allowed to remain at room temperature for 5 minutes, centrifuged, and read. If blocking antibodies were present, the cells would then clump.

Fisk and McGee[43] - See P. 53

Gelatin Blocking Test.- The gelatin solution was prepared by dissolving 10 g of gelatin and 1 g of di-sodium acid phosphate in 90 ml of distilled water. This was autoclaved for 15 minutes at 121 C. After sterilization, the pH was about 6.7. The solution could be kept at room temperature and heated before use. 1:10,000 merthiolate was added to avoid contamination.

The qualitative test - 1 drop of undiluted plasma or serum, 1 drop of 10 per cent gelatin solution, and 1 drop of oxalated blood of known Rh type were placed in a test tube. This was incubated at 37 C for 5 minutes and diluted with 2 ml of saline before reading. Fresh blood specimens must be used.

The quantitative test - the test was set up as for an agglutinin titration. Dilutions of the serum and the 2 per cent cell suspension were prepared in saline. 0.2 ml of the serum dilution and 1 drop of the 2 per cent cell suspension were added to the appropriate tube. After 30 minutes at 37 C the reactions were read. The tubes were then centrifuged, the saline removed, and replaced by 0.2 ml of a 3 per cent gelatin solution. An alternate method was to eliminate the removal of saline and add 0.1 ml of the 10 per cent gelatin solution to each tube. The material was shaken and reincubated at 37 C for 30 minutes. Before reading 0.2 ml of saline was added to each tube. This did not interfere with the reaction and eliminated pseudo-reactions.

Coombs[25] - See P. 53

<u>Direct Test for Blocking Antibodies</u>.- The red cells were freed from serum as completely as possible by washing 3 times in large volumes of saline. 2-5 per cent cell suspensions

were prepared in saline. Either 1 of 2 methods which gave parallel results may be used. If the test was carried out on a tile, 1 drop of the rabbit's anti-globulin serum was added to 1 drop of the cell suspension. The tile was rocked. If positive, the reaction occurred at room temperature in 5-10 minutes. The test may be carried out in the same way in test tubes.

Coombs[23] - See P. 54

Indirect Test.- Two drops of the serum to be tested and 2 drops of a 2 per cent washed red cell suspension of the appropriate type were added to small test tubes. After incubation at 37 C for 30 minutes, the sediment was examined microscopically. Two points in the technic were of particular importance. One was the use of a 2 per cent cell suspension. If too light the cells would not agglutinate. If too heavy, the reaction was more difficult to read. The second point was that 3 washings were necessary to remove all human globulin except that which was specifically absorbed onto the red cell surface. It was important to carry out the first part of the test at 37 C, but the second part showed equally good results at 37 C, 22 C, and 8 C.

Simmons - See P.

Methods of Preparing Sera Used as Antigens.- One method

was the alcoholic precipitation of globulin, which was then dissolved in saline for use. The second method was Proom's method[110] using alum-precipitated serum.

Haberman and Hill[55] - See P. 57

Elution of Rh Antibodies from Sensitized Erythrocytes.- The red cells of cord blood were washed 3 times in cold saline. After the last washing 1 ml of fresh saline was added to 0.25 ml of packed cells. The cells were resuspended and placed in a 56 C water bath for 5 minutes. The suspension was immediately centrifuged at 3,000 r.p.m. for 5 minutes, keeping the temperature at 56 C. The saline eluate was removed and recentrifuged to assure removal of all cells. The supernate was then tested for the presence of Rh agglutinins in the usual way by mixing 1 drop of the eluate with 1 drop of 2 per cent suspension of red cells in saline.

Chown[20] - See P. 58

Capillary Tube Method.- Whole blood or blood diluted up to 1:4 could be used. Capillary tubing with a 0.4 mm bore was cut into 0.8 mm lengths and sterilized. One end was dipped into an agglutinating serum and a column of about 2 mm allowed to enter. The same end was then dipped into the blood without any air bubbles intervening. The tube was inverted a couple of times to permit mixing. One end

was placed in a lump of plasticine at a 45° angle from the horizontal. The tube was incubated at 37 C for 15 minutes and read against a white background.

BIBLIOGRAPHY

1. Belkin, R.B. and Wiener, A.S., Proc. Soc. Exper. Biol. & Med., 1944,56:214

2. Berlin, R.B., Am. J. Clin. Path., 1947,17:233

3. Boorman, K.E. and Dodd, B.E., J. Path. & Bact., 1943, 55:329

4. Boorman, K.E., Dodd, B.E. and Mellison, P.L., J. Path. & Bact., 1945,57:157

5. Boorman, K.E., Dodd, B.E. and Morgan, W.T., Nature, 1945,156:663

6. Boyd, W.C., Unpublished experiments

7. Boyd, W.B., Commercial antisera - Unpublished experiments

8. Boyd, W.C., J. Exper. Med., 1946,83:401

9. Boyd, W.C., J. Exper. Med., 1946,83:221

10. Boyd, W.C., Southern J. Anthropology, 1947,3:32

11. Callender, S.T. and Paykoc, Z.V., Brit. J. Med., 1946, 1:119

12. Calvin, M., Evans, R.S., Behrendt, V. and Calvin, G., Proc. Soc. Exper. Biol. & Med., 1946,61:416

13. Cappell, D.F. and McFarlane, M.N., Lancet, 1946, :558

14. Carter, B.B., Am. J. Clin. Path., 1945,15:278

15. Carter, B.B. and Loughrey, J., Am. J. Clin. Path., 1945,15:575

16. Carter, B.B., Am. J. Clin. Path., 1947,17:646

17. Carter, B.B., J. Immunol., 1949,61:79

18. Castle, W.B., Wintrobe, M.M. and Snyder, L.H., Science, 1948,107:27

19. Chase, M.W., Proc. Soc. Exper. Biol. & Med., 1943,52:238

20. Chown, B., Am. J. Clin. Path., 1944,14:114

21. Coffin, G.S. and Kabat, E.A., J. Immunol., 1946,52:201

21a. Cohn, E.J., Oncley, J.L., Strong, L.E., Hughes, W.L. and Armstrong, S.H., J. Clin. Invest., 1944,23:417

22. Coombs, R.R.A., Mourant, A.E. and Race, R.R., Lancet, 1945,July 7:15

23. Coombs, R.R.A., Mourant, A.E. and Race, R.R., Brit. J. Exper. Path., 1945,26:255

24. Coombs, R.R.A. and Race, R.R., Nature, 1945,156:233

25. Coombs, R.R.A., Mourant, A.E. and Race, R.R., Lancet, 1946,1:266

26. Coombs, R.R.A. and Mourant, A.E., J. Path. & Bact., 1947,59:105

27. Darrow, R.R., Arch. Path., 1938,25:378

28. Davidson, I. and Toharsky, B., Am. J. Clin. Path., 1942,12:434

29. De Gowin, E.L., J. Lab. & Clin. Med., 1945,30:99

30. Diamond, L.K., New Eng. J. Med., 1944,230:817

31. Diamond, L.K., New Eng. J. Med., 1945,232:447

32. Diamond, L.K. and Abelson, N.M., J. Lab. & Clin. Med., 1945,30:668

33. Diamond, L.K. and Denton, R.L., J. Lab. & Clin. Med., 1945,30:821

34. Diamond, L.K. and Abelson, N.M., J. Clin. Invest., 1945,24:122

35. Diamond, L.K. and Abelson, N.M., J. Lab. & Clin. Med., 1945,30:204

36. Diamond, L.K., Quoted by Potter, E.L., The Yearbook Publishers Inc., 1947

37. Diamond, L.K., Quoted in Castle, Wintrobe and Snyder, Science, 1948,107:27

38. Dick, D.S., Brit. M.J., 1947,2:95

39. Drummend, R., Taylor, G.L. and Rice, J.T., Brit. M.J., 1945,1:584

40. Echer, E.E., Erlanson, E.W., MacFarlane, M. and Laipply, T.C., Am. J. Clin. Path., 1944,14:168

41. Fisk, R.T. and Foord, A.G., Am. J. Clin. Path., 1942, 12:545

42. Fisk, R.T. and Foord, A.G., Am. J. Clin. Path., 1944, 14:431

43. Fisk, R.T. and McGee, C.A., Am. J. Clin. Path., 1947, 17:737

44. Fox, J.P., Quoted by Freund, Ann. Rev. Microb., 1947, p. 302

45. Freund, J. and Bonanto, M.U., J. Immunol., 1944,48:325

46. Freund, J., Sommer, H.E., Walter, A.W., Science, 1945, 102:200

47. Freund, J., Thomson, K.J., Sommer, H.E., Walter, A.W. and Schenkein, E.L., Science, 1945,102:202

48. Freund, J. and Bonanto, M.U., J. Immunol., 1946,52:231

49. Freund, J., Ann. Rev. Microb., 1947,1:291

50. Freund, J., Stern, E.R. and Pisani, T.M., J. Immunol., 1947,57:179

51. Freund, J., Thomson, K.J., Sommer, H.E., Walter, A.W. and Pisani, T.M., Am. J. Trop. Med., 1948,28:1

52. Friedewalde, W.F., Science, 1944,99:453

53. Friedewald, W.F., J. Exper. Med., 1944,80:477

54. Gallagher, F.W. and Jones, L.R., J. Immunol., 1943, 46:9

55. Gallagher, F.W. and Jones, L.R., Proc. Soc. Exper. Biol. & Med., 1943,53:119

56. Gallagher, F.W. and Pillischer, G.P., Science, 1947, 105:344

57. Gurevitch, J., Polishuk, Z. and Hermoni, D., Am. J. Clin. Path., 1947,17:465

58. Haberman, S.V. and Hill, J.M., Texas State J. Med., 1944,40:182

59. Halbert, S.P., Mudd, S. and Smolens, J., Proc. Soc. Exper. Biol. & Med., 1945,60:17

60. Haldane, J.B.S., Ann. Eugen., 1943,11:333

61. Heidelberger, M. and Mayer, M.M., Science, 1944,100:356

62. Henle, W. and Henle, G., Proc. Soc. Exper. Biol. & Med., 1945,59:179

63. Henry, N.R. and Simmons, R.T., M.J. Australia, 1946, June 29:897

64. Hill, J.M., Haberman, S. and Orozco, A.V., J.A.M.A., 1945,128:944

65. Hill, J.M. and Haberman, S., Nature, 1948,161:688

66. Hill, J.M., Haberman, S. and Jones, F., J. Hemat. Sp. Issue No. 2 1948 p. 83

67. Homburger, F., Proc. Soc. Exper. Biol. & Med., 1946, 61:101

68. Jacobowicz, R. and Bryce, L.M., M.J. Australia, 1946, No. 23:740

69. Kabat, E.A., Wolf, A. and Bazer, A.E., J. Exper. Med., 1947,85:117

70. Kopeloff, L.M. and Kopeloff, N., J. Immunol., 1944, 48:297

71. Kopeloff, L.M. and Kopeloff, N., J. Immunol., 1947, 57:229

72. Kulka, A.M. and Hirsch, D., J. Immunol., 1944,50:127

73. Kulka, A.M. and Hirsch, D., J. Immunol., 1946,53:391

74. Krieger, V.T. and Weiden, S., Am. J. Clin. Path., 1948,18:572

75. Landsteiner, K. and Miller, C.P.Jr., J. Exper. Med., 1925,42:853

76. Landsteiner, K., Levine, P. and Janes, M.L., Proc. Soc. Exper. Biol. & Med., 1928,25:672

77. Landsteiner, K. and Wiener, A.S., Proc. Soc. Exper. Biol. & Med., 1940,43:223

78. Landsteiner, K. and Wiener, A.S., J. Exper. Med., 1941,74:309

79. Landsteiner, K. and Wiener, A.S., Proc. Soc. Exper. Biol. & Med., 1942,51:313

80. Lawler, S.D. and Van Loghem, J.J., Lancet, 1947, Oct. 11:545

81. Levine, P. and Stetson, R.E., J.A.M.A., 1939,113:126

82. Levine, P., Katzin, E.M. and Burnham, L., Proc. Soc. Exper. Biol. & Med., 1940,45:346

83. Levine, P. and Katzin, E.M., Proc. Soc. Exper. Biol. & Med., 1940,45:343

84. Levine, P., Am. J. Clin. Path., 1941,11:898

85. Levine, P., Burnham, L., Katzin, E.M. and Vogel, P., Am. J. Obst. & Gynec., 1941,42:925

86. Levine, P., Am. J. Obst. & Gynec., 1941,42:165

87. Levine, P. and Polayes, S.H., Ann. Int. Med., 1941, 14:1903

88. Levine, P. and Katzin, E.M., Proc. Soc. Exper. Biol. & Med., 1941,48:126

89. Levine, P., West. J. Surg., 1942,50:468

90. Levine, P., J. Pediat., 1943,23:656

91. Levine, P., J.A.M.A., 1945,128:946

92. Levine, P., Science, 1945,102:1

93. Levine, P., Am. J. Clin. Path., 1946,16:597

94. Levine, P. and Waller, R.K., J. Hemat., 1946,1:143

95. Levine, P. and Waller, R.K., Science, 1946,103:389

96. Levine, P., Blood, Special Issue, 1948,2:3

97. Van Loghem, J.J., Brit. M.J., 1947,2:958

98. Van Loghem, J.J., Brit. M.J., 1948,2:326

99. Lubinsky, H.H., Canad. M.A.J., 1946,54:285

100. McFarlane, M., Ann. Eugen., 1946,13:15

101. Mohn, J.F. and Witebsky, E., N.Y. State J. Med., 1948,48:287

102. Moloney, W.C., Brit. M.J., 1945,2:916

103. Mourant, A.E., Nature, 1945,155:542

104. McIvor, B.C. and Lucia, S.P., Proc. Soc. Exper. Biol. & Med., 1943,52:293

105. Morgan, I.M., J. Exper. Med., 1947,85:131

106. Neter, E., J. Immunol., 1936,30:255

107. Ottenberg, R., J.A.M.A., 1923,81:295

108. Polayes, S.H., Am. J. Dis. Child., 1945,69:99

109. Potter, E.L., The Yearbook Publishers, Inc., 1947

110. Proom, H., J. Path. & Bact., 1943,55:419

111. Race, R.R. and Taylor, G.L., Nature, 1943,152:300

112. Race, R.R., Taylor, G.L., Boorman, K.E. and Dodd, B.E., Nature, 1943,152:563

113. Race, R.R., Taylor, G.L., Ikin, E.W. and Prior, A.M., Ann. Eugen., 1944,12:206

114. Race, R.R., Nature, 1944,153:771

115. Race, R.R., Taylor, G.L., Ikin, E.W. and Dobson, A.M., Ann. Eugen., 1945,12:261

116. Race, R.R., Mourant, A.E. and Callender, S., Nature, 1946,157:410

117. Race, R.R., Mourant, A.E. and McFarlane, M., Rev. d'hematol., 1946,1:9

118. Race, R.R., Sanger, R.R. and Lawler, S.D., Nature, 1948,161:316

119. Race, R.R., Blood, Special Issue, 1948,2:27

120. Rice, W.G. and Watson, F.G., Am. J. Clin. Path., 1948,18:598

121. Sacks, M.S., Kuhns, W.J. and Valin, E.F., Am. J. Obst. & Gynec., 1947,54:400

122. Scherer, W.F., Am. J. Med. Sc., 1948,215:33

123. Simmons, R.T., Graydon, J.J., Jacobowicz, R. and Bryce, L.M., M.J. Australia, 1943, :496

124. Simmons, R.T. and Graydon, J.J., M.J. Australia, 1945,2:325

125. Simmons, R.T., Nature, 1946,158:486

126. Simmons, R.T., M.J. Australia, 1947, Mar.22:370

127. Speck, G. and Sonn, E.B., Am. J. Obst. & Gynec., 1945,49:273

128. Stratton, F., Ann. Eugen., 1945,12:250

129. Stratton, F., Nature, 1946,158:25

130. Sussman, L.N., Am. J. Clin. Path., 1947,17:643

131. Thomson, K.J., Freund, J., Sommer, H.E. and Walter, A.W., Am. J. Trop. Med., 1947,27:79

132. Unger, L.J., J. Lab. & Clin. Med., 1946,31:793

133. U.S. Government, National Institute of Health, Federal Security Agency, 1946

134. Waller, R.K., Am. J. Clin. Path., 1944,14:116

135. Wallerstein, H., Blood, Special Issue, 1948,2:170

136. Warren, J. and Hough, R.G., Proc. Soc. Exper. Biol. & Med., 1946,61:109

137. Warren, E.W. and Scholl, M.L., Am. J. Dis. Child., 1947,74:274

138. Wiener, A.S. and Peters, H.R., Ann. Int. Med., 1940, 13:2306

139. Wiener, A.S., Arch. Path., 1941,32:227

140. Wiener, A.S. and Forer, S., Proc. Soc. Exper. Biol. & Med., 1941,47:215

141. Wiener, A.S. and Landsteiner, K., Proc. Soc. Exper. Biol. & Med., 1943,53:167

142. Wiener, A.S., Science, 1943,98:182

143. Wiener, A.S., Proc. Soc. Exper. Biol. & Med., 1944, 56:173

144. Wiener, A.S., Sonn, E.B. and Belkin, R.B., J. Exper. Med., 1944,79:235

145. Wiener, A.S., Proc. Soc. Exper. Biol. & Med., 1945, 58:133

146. Wiener, A.S., Science, 1945,102:479

147. Wiener, A.S. and Sonn, E.B., J. Lab. & Clin. Med., 1945,30:395

148. Wiener, A.S., J. Lab. & Clin. Med., 1945,30:662

149. Wiener, A.S., J. Lab. & Clin. Med., 1945,30:457

150. Wiener, A.S., J. Lab. & Clin. Med., 1946,31:575

151. Wiener, A.S., Am. J. Clin. Path., 1946,16:477

152. Wiener, A.S., Am. J. Med. Technology, 1946,12:95

153. Wiener, A.S., Am. J. Dis. Child., 1946,71:14

154. Wiener, A.S., Am. J. Clin. Path., 1947,17:165

155. Wiener, A.S. and Hurst, J.G., Exper. Med. Surg., 1947, 5:285

156. Wiener, A.S. and Sonn, E.B., Am. J. Clin. Path., 1947, 17:67

157. Wiener, A.S. and Handman, L., Exper. Med. Surg., 1948,6:416

158. Witebsky, E., Anderson, G.W. and Heide, A., Proc. Soc. Exper. Biol. & Med., 1942,49:179

159. Witebsky, E. and Heide, A., Proc. Soc. Exper. Biol. & Med., 1943,52:280

160. Witebsky, E., Klendshoj, N.C. and McNeal, C., Proc. Soc. Exper. Biol. & Med., 1944,55:167

161. Witebsky, E. and Mohn, J.F., J. Exper. Med., 1945, 82:143

162. Witebsky, E., Mohn, J.F., Howles, D.J. and Ward, H.M., Proc. Soc. Exper. Biol. & Med., 1946,61:1

163. Witebsky, E., Rubin, M.I. and Blum, L., J. Lab. & Clin. Med., 1947,32:1330

164. Witebsky, E., Blood, Special Issue, 1948,2:66

AUTOBIOGRAPHY

BIRTH: May 5, 1917 Newark, New Jersey

PARENTS: Ralph Sommer
Eva H. Sommer

SCHOOLS: Public School, Brooklyn, New York 1931

Erasmus Hall High School, Brooklyn, New York 1935

Brooklyn College, Brooklyn, New York B.A., 1939

University of Pennsylvania, Philadelphia, Pa., M.A., 1940

POSITIONS: Research Bacteriologist - Children's Hospital of Philadelphia - 3 years 9 months

Technician - Thorndyke Institute, Boston City Hospital - 9 months

Research Assistant to Dr. Jules Freund - Public Health Research Institute, New York City, New York - 2 years 4 months

PUBLISHED PAPERS:

1. The Variation of Group C Hemolytic Streptococci
Morton, H.E. and Sommer, H.E., J. Bact., 1944,47: 123

Studies on Air-Borne Infections in a Hospital Ward

2. I. The Effect of Ultraviolet Light on Cross-Infection in an Infants' Ward
Sommer, H.E. and Stokes, J.Jr.

3. II. Effects of Ultraviolet Irradiation and Propylene Glycol Vaporization Upon the Prevention of Experimental Air-Borne Infection of Mice by Droplet Nuclei
Henle, W., Sommer, H.E. and Stokes, J. Jr., J. Pediat., 1942,21:569 and 577

4. The Administration of Sulfonamide Microcrystals by Inhalation
Harris, T.N., Sommer, H.E. and Chapple, C.C., Am. J. Med. Sc., 1945,205:1

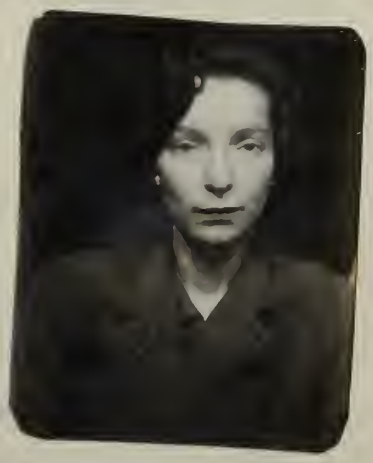
5. Immunization against Malaria: Vaccination of Ducks with Killed Parasites Incorporated with Adjuvants
Freund, J., Sommer, H.E. and Walter, A.W., Science, 1945,102:200

6. Immunization of Rhesus Monkeys against Malarial Infection (P. knowlesi) with Killed Parasites and Adjuvants
Freund, J., Thomson, K.J., Sommer, H.E., Walter, A.W. and Schenkein, E.L., Science, 1945,102:202

7. Immunization of Ducks against Malaria by means of Killed Parasites with or without Adjuvants
Thomson, K.J., Freund, J., Sommer, H.E. and Walter, A.W., Am. J. Trop. Med., 1947,27:79

8. Immunization of Monkeys against Malaria by means of Killed Parasites with Adjuvants
Am. J. Trop. Med., 1948,28:1

9. Antibody Formation and Sensitization with the Aid of Adjuvants
Freund, J., Thomson, K.J., Hough, H.B., Sommer, H.E. and Pisani, T.M., J. Immunol., 1948,60:383



ABSTRACT

The Use of Adjuvants in the Production of Rh Antisera in Animals

The purpose of this investigation was to determine whether the avidity and strength of Rh antisera produced in animals could be increased by incorporating the antigen into a water-in-oil emulsion.

The discovery of a new antigen present in human erythrocytes was indicated in 1940 by 3 lines of investigation. Landsteiner and Wiener reported an agglutinin produced in rabbits injected with Macacus rhesus erythrocytes. Identical agglutinins were observed in 2 cases of intra-group transfusion reactions by Wiener and Peters, and in a woman who gave birth to an erythroblastotic infant by Levine and Katzin.

The need for Rh antisera has taken its place next to that for anti-A and anti-B blood grouping sera. The clinical situations where it is necessary to know the Rh types are in transfusions, pregnancies, and infants with evidence of erythroblastosis. Prognosis for future pregnancies of women with erythroblastotic infants may be made by determining the homo- or heterozygosity of the

father. Rh typing is also useful in medico-legal problems and in familial and anthropological studies.

Most of the antisera used at present are obtained from mothers of erythroblastotic infants, persons who have received multiple transfusions of blood incompatible for the Rh-Hr antigens and male volunteers. The most potent sera are obtained from the first group where there is a constant antigenic stimulus over a long period of time.

Numerous workers have attempted to produce usable Rh antisera in rabbits and guinea pigs by injecting them with rhesus erythrocytes, human erythrocytes, or stroma or extracts prepared from the latter. The general conclusion arising from such studies is that the Rh factors are poor antigens when administered in the usual way.

Increasing use has been made of adjuvants to enhance antigenic stimulus. When the antigen is suspended in the water phase of a water-in-oil emulsion, it is released more slowly over a longer period of time. This method of immunization has been successful with a number of substances, including such weak antigens as alcoholic extract of brain, histaminazo protein and ragweed pollen.

Since the most potent Rh antisera in humans are produced in mothers of erythroblastotic infants, and since

the adjuvant method of immunization also exposes the reactive organism to the antigenic stimulus over a long interval, this method was applied in the present investigation.

Two experiments were carried out. In both the adjuvants used were Falba, Bayol F, and tubercle bacilli. The antigen was suspended in the water phase of a water-in-oil emulsion. Rhesus erythrocytes and rhesus stroma in emulsion were injected intraabdominally into guinea pigs and rabbits in the first experiment. Human Group O, MN, Rh -/- erythrocytes and stroma in saline were injected intraabdominally, and in emulsion intraabdominally and subcutaneously into guinea pigs in the second experiment. Sera were obtained by cardiac puncture at 0, 1, 2, 3, 4, 6, 8, 10, and 12 week intervals after initiation of the experiment. In Experiment I they were examined for agglutinins by titrating in steps of 10 using two 2 per cent suspensions of Rh -/- red cells and 2 of Rh negative erythrocytes. Sera from Experiment II were examined for agglutinins in the same way, absorbed with Group O, MN, Rh negative erythrocytes, and the absorbed sera tested for anti-Rh antibodies with the same four 2 per cent cell suspensions. When tested for agglutinins and blocking antibodies, the cells were suspended in 0.9 per cent

saline and in 30 per cent bovine albumin respectively. In this study, an adequate Rh antiserum was defined as one that showed at least a 2 step difference in strength of reaction between Rh -/- and Rh negative cells. These sera that showed a 1 or 1 1/2 step difference were called weak.

Agglutinins produced in guinea pigs and rabbits to rhesus erythrocytes and stroma suspended in emulsion disappeared after standing in the refrigerator for a month and a half after the original titrations. This seemed to indicate that Rh agglutinins, if present, were too weak to be useful.

Of the 3 groups of guinea pigs injected with human Group O, MN, Rh -/- erythrocytes, adequate agglutinins were produced only in Group 1 in which plain antigen had been injected intraabdominally. Six of the 10 guinea pigs produced adequate agglutinins, and 9 produced suggestive antibodies. Group 2, injected subcutaneously with the antigen-emulsion, produced no Rh agglutinins. One animal in Group 3, which received the antigen in emulsion intraabdominally, produced suggestive antibodies.

Of the 3 groups injected with stroma prepared from the above red cells, 1 animal in Group 4, which received the plain stroma intraabdominally, produced adequate agglu-

tinins and 1 showed suggestive agglutinins. One animal in Group 5, which received the antigen-emulsion subcutaneously, showed suggestive antibodies. Group 6, which received the emulsion intraabdominally, behaved similarly to Group 5.

The time of appearance of specific Rh agglutinins differed markedly from the peak of general agglutinin production. Six of the 7 adequate Rh antisera were obtained from guinea pigs 1 week and one 2 weeks after the first injection of antigen. Antisera obtained 4, 6, 8, 10, and 12 weeks after injections were started showed no Rh agglutinins. Injections of the antigen were continued through the fifth week. Of the 13 guinea pigs producing suggestive Rh agglutinins, 1 produced the antibodies after the first and second week of injection, 1 after the first and not subsequently, 10 two weeks after, and 1 the fourth week after injections were started.

When sera of the 6 experimental groups had been tested for the presence of general anti-human Group O, MN agglutinins, and for their strength, when present, by titration in steps of 10, all 6 groups showed essentially the same results. The titer was the same when tested against the 4 separate suspensions of erythrocytes, 2 Rh + and 2 Rh negative. Sera obtained 1 and 2 weeks after the

first injection, which contained all 7 of the adequate and 13 of the 14 suggestive Rh antisera, showed a titer to the erythrocytes as a whole of undiluted to 1:1,000, the majority at 1:100. The peak of general agglutinin production to the whole cell or stroma occurred from the fourth to sixth week, primarily at the former. The peak titer in no case exceeded 1:10,000. There was no relationship between titer and later demonstration of specific Rh agglutinins upon absorption.

Of the technics used to determine the presence of Rh agglutinins, namely, absorption of non-Rh agglutinins with Group O, MN, Rh negative erythrocytes, and dilution of the sera, only the former method was effective. In no instance did dilution of the serum in steps of 10 produce either adequate or weak agglutinins. No prezone phenomena was observed.

The sera were also tested for the presence of Rh blocking antibodies. None were observed after absorption of the antisera with Group O, MN, Rh negative red cells.

The unabsorbed sera from the first and second week bleedings were titrated in steps of 10 against red cells suspended in 30 per cent bovine albumin. There was no difference in titer between sera when tested against cells suspended in 0.9 per cent NaCl and the albumin.

Since agglutinin production was so poor and no blocking antibodies were observed, the more delicate Coombs antibodies were not investigated.